Is the circulating UK Bordetella pertussis population evolving to evade vaccine-induced immunity?

Sealey, Katie

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Is the circulating UK *Bordetella pertussis* population evolving to evade vaccine-induced immunity?

Katie Lynn Sealey

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Abstract

Bordetella pertussis is the causative agent of whooping cough (or pertussis) that has become resurgent worldwide. Resurgence has been linked to the introduction of acellular pertussis vaccines (ACVs) and the evolution of *B. pertussis* away from vaccine-induced immunity. In 2012, the United Kingdom suffered a major pertussis outbreak. I conducted whole genome sequencing and genomic analysis of 95 strains including those isolated from the UK outbreak and demonstrated that although large-scale genetic changes in *B. pertussis* were not the cause of this outbreak, vaccine-antigen encoding genes are evolving at higher rates than other surface protein-encoding genes, this difference becoming more pronounced since the introduction of the ACV in the UK.

The dramatic increase in the frequency of pertactin (Prn)-deficient strains worldwide is possibly in response to vaccine-mediated selection pressure. However, just one Prn-deficient strain was identified among the UK outbreak strains. Prn expression and IgG binding to *B. pertussis* obtained with post-vaccine sera was determined by flow cytometry and compared between pre-outbreak and outbreak strains, but no significant differences were observed. However, a positive correlation between Prn expression and post-vaccination induced IgG binding to strains was identified, supporting the idea that strong immunological selection pressure is exerted on Prn and this is playing a role in the evolution of *B. pertussis*. The rapid evolution of vaccine-antigen encoding genes raises serious concerns regarding the ability of current vaccines to control pertussis.

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1

Introduction

1.1 Bordetella pertussis Infection and Disease.

Bordetella pertussis is a gram-negative coccobacillus that is the leading cause of the respiratory tract disease whooping cough, or pertussis, in humans. Pertussis was a major global cause of mortality and morbidity, particularly in infants until the introduction of vaccination against pertussis resulted in a significant decrease in disease incidence among vaccinated populations. However, pertussis is still considered the leading cause of death among bacterial vaccine-preventable diseases, with approximately 140 000 reported cases and 89 000 deaths worldwide in 2014 (WHO, 2014). Reported cases underestimate the true burden of disease with estimates of the actual number of annual cases numbering in the millions. During the last 10-15 years a number of countries have experienced a resurgence of pertussis despite continued high levels of pertussis vaccination raising concerns over the ability of vaccination to continue to control this disease.

B. pertussis is acquired through infected droplets from other hosts. The bacteria display a strong tropism for the cilia of the respiratory mucosa and this represents the major site of infection (Paddock *et al.,* 2008). Colonisation is followed by proliferation on the ciliated mucosal surface resulting in ciliostasis, damage to the respiratory epithelium, induction of mucus release and an inflammatory influx into the lumen of the respiratory tract. This initial stage of infection is characterised by mild symptoms similar to those of a common cold; including rhinorrhea, mild cough and sneezing.

Infection typically progresses to the 'paroxysmal phase' characterised by the classic symptoms of pertussis including severe paroxysmal coughing. Paroxysms can occur multiple times each hour, every hour over several weeks leaving the host exhausted and gasping for air. This inhalation following coughing produces the distinctive 'whoop' sound of pertussis. The cause of paroxysmal cough during pertussis is unknown. For a long time pertussis was considered a disease only of infants and children but it is now recognized that all ages are at risk of infection, but that infants are at greatest risk of severe disease and death and in which complications are common including seizures and choking. A pause of breath leading to irregular breathing, known as apnoea, is more common in

neonates (Shojaei *et al*., 2014). Death is associated with pulmonary hypertension resulting in cardiac failure (Paddock *et al*., 2008), and occurs mainly in previously healthy infants. In these cases the combined effects of apnoea and pneumonia result in hypoxaemia which in turn leads to pulmonary vasoconstriction. The pronounced leukocytosis that is a hallmark of severe pertussis increases vascular resistance. The overall effect of these pathologies is pulmonary hypertension leading to cardiac failure and shock (Paddock *et al.,* 2008).

Older children and adolescents also suffer pertussis. Anecdotally, pertussis in these age groups was described as milder than in infants. However, systematic study of disease in adults and adolescents reveals that disease resembling classic whooping cough is uncommon (Cherry and Paddock, 2014). It is probable that pertussis has been largely underestimated in this age category due to misdiagnosis (Masseria and Krishnarajah, 2015) and the true burden of disease in adults is uncertain.

During convalescence coughing gradually subsides and bacteria are cleared from the respiratory tract by adaptive immune responses (see below).

Table 1.1 Three stages of whooping cough infection

Stage of infection	**Pathology/symptoms**
Catarrhal	Adherence of bacteria to cilia, bacterial multiplication, resistance to clearance by innate immunity. Symptoms similar to those of a common cold. • Profuse and mucoid rhinorrhea • Malaise, fever, mild cough, sneezing and anorexia
Paroxysmal	Cause of cough unclear, possibly due to pertussis toxin. Severe paroxysmal coughing, often followed by classic "whoop" on inhalation, breathlessness, exhaustion, vomiting, choking, breaking ribs • Life threatening in infants with underlying cardiac or pulmonary disease • Can lead to neurological complications
Convalescent	Bacterial clearance by adaptive immunity.

1.2 Epidemiology and vaccination

Gaining an accurate picture of *B. pertussis* epidemiology is difficult. Classically described whooping cough affects mainly infants and children, and thus the disease was considered to be one largely of childhood. Over time, it was realised that mild or atypical pertussis is common, and importantly, that *B. pertussis* is frequently detected in adults with chronic cough (Cherry and Paddock, 2014). Thus, *B. pertussis* is probably endemic throughout the population. Until the middle of the twentieth century pertussis was a major cause of disease in infants. During the 1940's in the UK there were 980 000 notified cases (Fig 1.1), and in the same period in the USA there were an average of 175 000 cases per year (CDC, 2015). The disease pattern was cyclical with peaks at 3-4 year intervals. The introduction of vaccination (see below) led to significant reductions in cases of pertussis. The changing epidemiology of pertussis in recent years is discussed in later sections (see section 1.4.1, page 17).

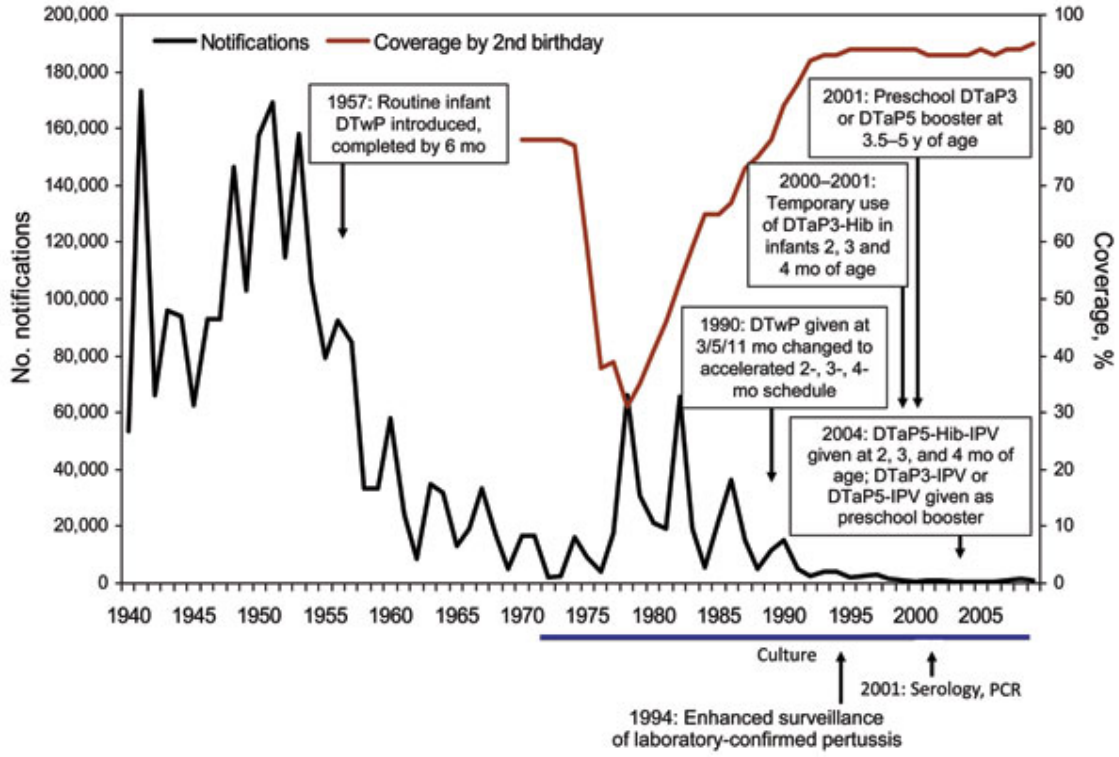

Fig.1.1 Whooping cough notifications and vaccine coverage in England and Wales from the years 1940-2010 Changes to the vaccine used, vaccine schedule and diagnostic methods used are shown. (Campbell *et al*., 2012).

The first pertussis vaccines were developed during the 1930's and implemented in most developed countries during the 1940's and 1950's (Cherry, 1996). These were whole cell vaccines (WCVs) comprised of chemically-killed bacteria. Often administered in combination with diphtheria and tetanus toxoids, a full primary course of vaccination comprised three injections. Japan started using a WCV in 1949, the Netherlands and Sweden began using the WCV in 1953 (King *et al*., 2013). The UK appeared reluctant to introduce vaccination. This probably stemmed from conflicting data regarding the efficacy of WCVs. Numerous different WCVs were available and there were significant differences in their composition and resulting efficacy. The development of a mouse intra-cerebral challenge model in which vaccination was protective allowed the development of measures of vaccine potency and standardization of WCVs. In 1957, the UK introduced a vaccine containing diphtheria, tetanus and whole-cell pertussis (DTwP) (Amirthalingham *et al.*, 2013). Initially vaccination was at 3, 5 and 11 months of age. Following the introduction of vaccination there was a very large reduction in the incidence of disease, demonstrating that WCVs were effective at preventing classic pertussis, i.e. whooping cough in infants and children, which was certainly the main recognised form of *B. pertussis* infection at that time. The introduction of vaccination globally has been a major success. Monitoring by WHO recorded around 140 000 cases of pertussis globally in 2014, resulting in 89 000 deaths (WHO, 2014). Although this probably represents under-reporting it nevertheless signifies a tremendous reduction in disease burden compared to the pre-vaccination era.

However, WCVs were considered 'reactogenic', causing side effects including fretfulness and injection site soreness and swelling. In particular more serious complications began to be associated with pertussis vaccination including seizures, other neurological sequelae and even sudden infant death, (Cherry, 1992). The evidence for and against 'pertussis vaccine encephalopathy' has been reviewed extensively (for example (Mattoo and Cherry, 2005)). It is likely that the apparent linkage between infantile epilepsy, sudden infant death syndrome and pertussis vaccination is due to temporal association, with no cause and effect relationship. This is similar to the measles, mumps, and rubella (MMR) vaccination and autism controversy in 2002 (Farrington and Miller, 2005). Unfortunately a growing public distrust of the vaccines led to dramatic decreases in vaccination coverage. For example, in the UK coverage fell from 79% to 31% between 1973-8, contributing to a large-scale epidemic from 1977-1979 (Amirthalingham *et al.*, 2013) (Fig.1.1) as well as a further two outbreaks in 1982 and 1985 (Baker, 2003). Eventually, public trust in the vaccine was restored when studies showed that the benefit from protection provided by the

vaccine outweighed its risks and disease returned to low incidence levels. In 1990 the vaccine schedule changed to an accelerated schedule of 2-, 3- and 4-month primary doses to achieve protection against pertussis at an earlier age and to aid greater coverage with a full course of vaccination (Amirthalingham *et al.*, 2013).

Concerns about the safety of WCVs prompted the development of a second generation of pertussis vaccines, the acellular vaccines (ACV). These comprise 1 to 5 highly purified *B. pertussis* proteins: pertussis toxin (PT), filamentous haemagglutinin (FHA), pertactin (Prn) and fimbrial proteins 2 and 3 (Fim2 and 3). A number of mono-, bi- and multi-antigen ACVs have been developed. The most commonly used contain either PT, FHA and Prn, or all five antigens, but all contain PT. Detailed reviews of these vaccines are available elsewhere (for example see (Decker and Edwards, 2000). Their efficacy to prevent pertussis appears to be slightly lower than that of WCVs, but they have better safety profiles than WCVs in terms of causing adverse reactions. Importantly, the immune responses stimulated by WCV and ACV differ, being Th1/Th17 biased vs Th2/Th17 biased respectively (Ross *et al*., 2013). The consequences of this for host immunity-pathogen interactions are unknown, but may have contributed to the resurgence of pertussis (see below). Initially introduced as booster vaccinations following primary immunisation with WCV, many developed countries switched to ACVs for the entire vaccination course at the end of the 1990's and the early 2000's. Much of the developing world continues to use the cheaper WCVs.

During the 1990's, a picture was emerging of waning immunity among adults being important for maintaining disease transmission. In the UK, there was concern that the accelerated primary schedule would lead to early waning of vaccine-induced immunity in pre-school years and transmission of pertussis from this age group to unvaccinated infants. When given as boosters, WCVs were very reactogenic. Thus, in 2001 in the UK, an ACV was used for the pre-school booster vaccination and from 2004, ACVs replaced WCVs for the entire vaccination schedule (Amirthalingham *et al.*, 2013). It is estimated that switching from a three- to four-dose schedule provided 46% additional effectiveness (95% CI:7 to 71%) with effectiveness being 95.3% (95% CI: 91.9 to 97.2%) with monitoring for cases up to 7 years (Campbell et al., 2012).

1.3 ACV antigens, their role in pathogenesis and regulation of expression in B. pertussis

1.3.1 Acellular vaccine antigens

The antigens that comprise the ACVs are *B. pertussis* proteins selected due to their perceived role in *Bordetella* virulence. It was reasoned that induction of immune responses against these immunogenic proteins would target processes important for *B. pertussis* infection and thus prevent disease. Pertussis toxin is an AB_5 exotoxin in which the B subunit (S2, comprising PtxB-E) binds to host cells to deliver the enzymatically active A subunit into the host cell. This subunit (S1, the PtxA protein) is an ADP-ribosylase that transfers ADP-ribose to host cell αi subunits of G proteins, inhibiting G protein activity. This allows unregulated adenylate cyclase activity increasing the intracellular concentration of cyclic adenosine monophosphate (cAMP) with subsequent interference with intracellular signaling. Many effects have been associated with the action of PT including a loss of electrolytes and fluids and secretion of mucus. PT also reduces neutrophil recruitment to the site of infection (Andreasen and Carbonetti, 2008; Carbonetti *et al*, 2007). The overall effect of PT appears to be delayed recruitment of immune cells to the site of infection thus aiding the establishment of infection (Carbonetti, 2015). The remaining antigens have adhesin activity and are thought to enable *B. pertussis* to adhere to host cells and tissues during infection. Fimbrial antigens 2 and 3 (Fim2 and Fim3) form distinct fimbrial appendages from which the fimbrial tip adheres to sulfated sugars that are found throughout the mammalian respiratory tract (Geuijen *et al.,* 1998). Filamentous haemagglutinin (FHA) is a large, dominant adhesin involved in binding to the ciliated epithelium (Paddock *et al.*, 2008) and evasion of complement (Jongerius *et al.*, 2015). Pertactin (Prn) is an outer membrane protein of the auto-transporter class that mediates binding to human epithelial cells (Otsuka *et al.*, 2012) as well as playing a role in resisting neutrophil mediated clearance (Inatsuka *et al*., 2010).

1.3.2 The two-component virulence regulator

BvgAS is a two-component system that is a global regulator of gene expression in *B. pertussis* (Cotter and Jones, 2003). Two component systems sense environmental stimuli and coordinate a response through the regulation of gene expression profiles. BvgS is the membrane sensor histidine-kinase, and BvgA is the partner phosphate-accepting response regulator. BvgS responds to environmental cues, which initiate a multi-step pathway leading to the phosphorylation of BvgA. Upon phosphorylation, BvgA directly interacts with promoter regions of target genes whereby transcription of genes is initiated or

repressed (Willems *et al.*, 1990; Chen *et al*., 2010; Kinnear *et al*., 2001). In *B. pertussis* BvgAS regulates the expression of approximately 1000 genes (A. Preston, personal communication) and in doing so directs *B. pertussis* between distinct phenotypes, a phenomenon known as modulation. Lacey (1960) was the first to detect this modulation in *B. pertussis* and identified three phenotypic phases: X mode, C mode and I mode, now known as Bvg^+, Bvg^- and Bvg^i, respectively (Cotter and Jones, 2003).

The Bvg^+ phase is characterised by BvgA binding to the promoter regions of virulence genes to increase transcription and thus production of 'virulence factors' such as PT, fimbriae, adenylate cyclase and FHA involved in pathogenesis. These virulence-activated genes (*vag*) can be classed as early- or late-activated genes (Jones *et al.*, 2005), for example, *ptxA-E* (that encode the PT subunits) is classed as late activated, however, *fhaB* is classed as an early Bvg^+ activated gene (Jones *et al.*, 2005). The Bvg^+ phase is active at 37°C, the temperature of the human host. During this phase, BvgA also represses genes known as the virulence repressed genes (*vrg*). It has been proposed that during Bvg^+ phase, a gene designated *bvgR* (Bvg repressor), is activated by the Bvg system and transcribed, producing a repressor protein responsible for the binding at *cis*-repressive sites in the promoter regions of *vrgs* (Merkel *et al*., 1995, 1998).

The Bvg^- phase is induced by a temperature change from 37°C to 25°C, or chemical modulators such as SO_4^{2-} ions at 50mM or increased nicotinic acid concentration. The Bvg^- phase is characterised by the activation of virulence repressed genes (*vrg*) and reduction in expression of virulence genes, due to the absence of phosphorylated BvgA and the lack of expression of BvgR. A definitive role for the Bvg^- phase in *B. pertussis* biology is yet to be determined. It is not necessary for persistence of pertussis infection in the mouse model but may be required for effective infection (Martinez de Tejada *et al.,* 1998) via transmission and effective colonization, as studied in mice (Martinez de Tejada., 1998; Locht *et al*., 2001).

The third phase is Bvg^i, an intermediate stage where the BvgAS system is neither completely activated or inactivated, and expression of other genes is induced such as *bipA*, encoding a putative outer membrane ligand binding protein, while a number of Bvg-repressed and activated genes are not expressed (Stockbauer *et al*., 2001; Jones *et al*., 2005; Decker *et al*., 2012). Very little is known about this Bvg^i phase, due to the overlap with both Bvg^+ and Bvg^- phases. However, in *B. bronchiseptica*, the Bvg^i phase has been suggested to be involved in early stages of infection, biofilm formation and transmission (Decker *et al*., 2012; Irie *et al*, 2004;Vergara-Irigaray *et al,* 2005). Cummings *et al* (2006) used microarrays to compare the gene expression profiles of the Bvg^+, Bvg^- and Bvg^i

phases of a *B. pertussis* clinical strain (GMT-1) and RB50, a *B. bronchiseptica* strain isolated from a rabbit. There were 117 genes expressed only in the Bvg^+ phase in *B. pertussis* GMT-1 genes, of which 71% were also expressed in *B. bronchiseptica,* suggesting the Bvg^+ phase in both these species may have a similar role (Cummings *et al.*, 2006), and gives a strong indication that *B. pertussis* inherited this system from the *B. bronchiseptica*-like ancestor. Those genes found to be specific to *B. pertussis* Bvg^+ phase included those encoding PT and its associated secretion system. Contrary to this, the *B. pertussis* and *B. bronchiseptica* Bvg^- phase shared very little similarity in gene expression. *B. pertussis* expressed a total of 34 Bvg^- phase-specific genes, as opposed to 229 genes in Bvg^- phase *B. bronchiseptica.* Only 5% of these *B. bronchiseptica* genes were shared with *B. pertussis.* These genes may have been lost during the evolution of *B. pertussis* as they may not have been required for survival in the human respiratory tract. *B. bronchiseptica* has a much wider host range and thus may need a wider variety of genes to be expressed for survival. Two distinct classes of Bvg^i phase were described in *B. bronchiseptica,* one class in which 69 genes were maximally expressed, and another in which 193 genes were maximally expressed. In contrast, for *B. pertussis* only 30 Bvg^i phase-specific genes were identified, including *bipA*. Cummings *et al* (2006) concluded that this Bvg^i phase in *B. pertussis* was likely to have been inherited from its ancestor. Cummings and colleagues (2006) also identified differences in gene expression between two *B. pertussis* strains, the laboratory-adapted Tohama I and GMT 1, highlighting the ability of *B. pertussis* to alter its phenotype, possibly in response to the different environment these strains occupy (laboratory and human, respectively). *B. pertussis* adopts the Bvg+ phase when grown at 37°C and modulates to the Bvg- phase at growth temperatures below ca. 27°C. Thus it is often reported that *B. pertussis* will be in the Bvg+ phase within the human respiratory tract but it is not clear what the temperature of respiratory tract may be in individual hosts during the course of an infection. The upper airways are likely to be cooler than the lungs. Thus it is possible that Bvg activity may vary depending on the infection site in the respiratory tract.

Additional mechanisms affecting *B. pertussis* virulence gene expression have been described. The promoters of *fim2* and *fim3* contain a homo-polymeric cytosine tract. The length of this tract varies between strains of *B. pertussis* and it is proposed that the length of the tract can vary via slip-strand misparing during DNA replication (Willems *et al*., 1990; Vaughan *et al.,* 2014). Strains that did not express Fim3 had shorter tracts (by 1-5 Cs) than those that did (Willems *et al.*, 1990) suggesting that phase variation of fimbrial

antigens was determined by the length of the poly-C-tract. In support of this, insertion or deletion of a C within the tract affected the binding capability and positioning of BvgA with respect to RNA polymerase (RNAP), (Chen *et al*., 2010). Recently, Vaughan and colleagues (2014) analysed whole genome sequence (WGS) data from 22 UK clinical strains that enabled identification of the total population of poly-C tract lengths of each strain, and correlated this with the level of expression of Fim2 and Fim3. Multiple populations with different polyC tract lengths of both *fim2* and *fim3* were observed within a single culture. With this ability to alter the binding capabilities of BvgA, and the dynamics of the *bvg* system, *B. pertussis* has an incredibly efficient and effective process for inducing phenotypic fluidity and adaptation to its environment.

1.4 Phylogeny and evolution of the Bordetellae

1.4.1 The Bordetella *species*

The genus *Bordetella* comprises eight validly named species (von Wintzingerode *et al*., 2001), and one that still awaits formal description, '*Bordetella ansorpii*' sp. nov. (Ko *et al*., 2005; Fry *et al*., 2007). The genome of at least one strain of each *Bordetella* species has been sequenced and thus comparisons of genome size and content are possible (Table 1.2) (Parkhill *et al*., 2003). *B. pertussis, B. parapertussis* and *B. bronchiseptica* make up the "classical *Bordetella"* group and infect exclusively mammals. *B. bronchiseptica* infects a wide variety of mammals, including swine, leading to atrophic rhinitis, and dogs causing kennel cough which can lead to pneumonia and death (Mattoo *et al*., 2005). *Bordetella bronchiseptica* can also infect immunocompromised humans and cause respiratory symptoms, leading to death in some cases (Ner *et al.*, 2003; Spilker *et al.,* 2008; Garcia-de-la-Fuente *et al.*, 2015). However, generally, *B. bronchiseptica* infections in humans are uncommon (Bjornstad and Harvill, 2005).

Two lineages of *B. parapertussis* have been described, one isolated from sheep, and another from humans. *B. parapertussis* can cause whooping cough but it is thought to be a more rare, and cause milder disease, than *B. pertussis* (Heininger 1994). Recent data have shown that *B. parapertussis* infections are more common than previously thought as Koepke *et al* (2015) found that *B. parapertussis* infections contributed to cases thought to be caused by *B. pertussis* during an outbreak of pertussis in Wisconsin. As mentioned above, *B. pertussis* is strictly a pathogen of humans and has not been found in any other host or environment, which may explain why it has the smallest gene content out of the "classical *Bordetella"*.

Not as much is known about the remaining *Bordetella*, although most have been isolated from humans. *Bordetella trematum* has been associated with wounds, ulcers, ear infection, bacteraemia, fever and vomiting in humans (Vandamme *et al*., 1996; Saksena *et al.*, 2015; Almagro-Molto *et al.*, 2015), *B. hinzii* has been isolated from rodents, poultry and immunocompetent and immunocompromised humans, (Vandamme *et al*., 1995; Jiyipong *et al.*, 2013; Palacián Ruiz *et al.*, 2013; Hristov *et al*., 2012) and the two reports of '*B. ansorpii*' involved isolation from an epidermal cyst and an immunocompromised host (Ko *et al*, 2005; Fry *et al*., 2007). *Bordetella avium* is an important bird pathogen that infects the upper respiratory airways and causes turkey coryza (Register & Yersin, 2005), and can infect humans (Harrington *et al.*, 2015), but such reports are rare. *Bordetella holmesii* has been isolated from the blood and nasopharynx of immunocompromised humans (Nijamkepo *et al.*, 2000). Russell *et al* (2001) detected *B. holmesii* infection by DNA sequencing from an individual who had severe pulmonary fibrosis with pertussis-like disease. There are other reports of pertussis-like disease due to *B. holmesii* (Pittet *et al.,* 2014) but the incidence of *B. holmesii* infection is unknown. There have been reports of misdiagnosis of *B. holmesii* cases from USA, Canada and France. Both *B. holmesii* and *B. pertussis* contain identical repeat elements that are targets for PCR-based diagnosis, thus infection from *B. holmesii* could have been mistaken for *B. pertussis* disease (Mazengia *et al*., 2000; Guthrie *et al*., 2010; Njamkepo *et al*., 2011). Finally, *B. petrii* appears to be the only species of *Bordetella* that has been found in the environment such as soil, grass roots and marine sponges, as well as from the respiratory tract of patients including those with cystic fibrosis and sinusitis (von Wintzingerode *et al*., 2001; Nagata *et al.*, 2015).

Table 1.2. Genome size, mean G+C content and host of the nine *Bordetella* species

Species	Genome Size (Mbp) (approx.)	Mean G + C content (%)	Hosts
B. holmesii	3.8	61.69	Human
B. pertussis	4.1	68.12	Human
B. parapertussis	4.8	68.43	Human
B. bronchiseptica	5.4	68.49	Rabbit, other mammals including human
B. parapertussis	4.9	68.15	Ovine
B. hinzii	4.9	65.44	Human
B. avium	3.7	61.58	Turkey, human (rare)
B. trematum	4.4	63.55	Human
'*B. ansorpii*'	6.2	64.26	Human
B. petrii	5.3	65.48	Environment, human

1.4.2 Phylogeny and evolution

Phylogenetic analysis using 16S rRNA gene sequences from the nine *Bordetella* species highlights their genetic relationship (Fig.1.2). *B. holmesii* is very closely related to *B. pertussis* as shown by previous observations (Weyant *et al*., 1995). The phylogenetic tree shows *B. hinzii, B. avium* and *B. trematum* as being closely related to each other and they may have arisen from a *B. petrii*-like ancestor. Gross and colleagues (2008) carried out sequence analysis of the genome of *B. petrii* and found proteins related to virulence of pathogenic bordetellae, as well as large genomic islands which may contribute to its metabolic ability in the environment. Due to the ability of *B. petrii* to occupy both environmental and mammalian niches, it was suggested that this species could be the

intermediate evolutionary state between environmental and pathogenic bordetellae such as *B. pertussis*.

1.4.3 B. bronchiseptica, B. pertussis *and* B. parapertussis: *the 'classical' bordetellae*

The phylogenetic tree (Fig, 1.2) shows clustering of the "classical bordetellae" (Diavatopoulos *et al*, 2005). Ovine *B. parapertussis* clusters with the *B. bronchiseptica* strain isolated from a rabbit, as may be expected due to their non-human hosts. The human *B. parapertussis* clusters near the human derived *B. pertussis* and *B. holmesii* strains. The dendrogram suggests that *B. pertussis* has diverged from *B. bronchiseptica.*

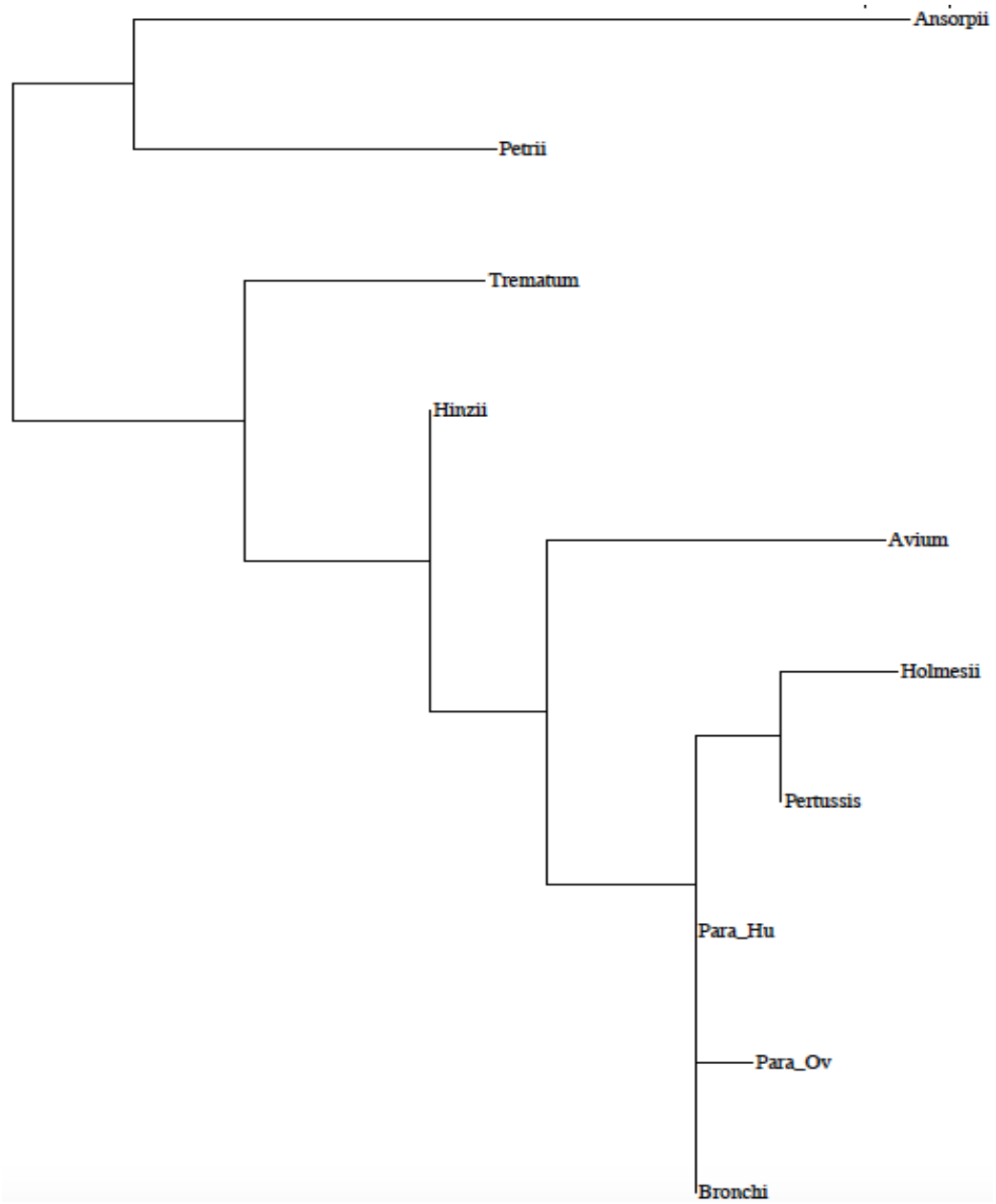

Fig. 1.2 The genetic relationship between the nine *Bordetellae* species based on 16S rRNA gene sequences. Tree was constructed in Seaview using PhyML. Human *B. parapertussis*: Para_Hu. Ovine *B. parapertussis*: Para_Ov.

As noted in Table 1.2, the genome sizes of these *Bordetella* are distinct, with *B. bronchiseptica* having the largest of ca. 5.4 Mbp compared to *B. parapertussis* ca. 4.8 Mbp and *B. pertussis* ca. 4.1 Mbp. Evidence suggests that *B. pertussis* and *B. parapertussis* evolved independently from a *B. bronchiseptica*-like ancestor via genome decay (Parkhill *et al*., 2003; van der Zee *et al*., 1997; Diavatopoulos *et al*., 2005). In support of this, progressive gene loss over time from *B. pertussis* has been shown (King *et al*., 2010; Park *et al*., 2012). This has been suggested as a way of adapting to the human host, by loss of genes not necessary for establishment in non-human environments, whilst retaining those essential for virulence (Bouchez *et al*., 2008). In *B. pertussis* gene loss and rearrangement occurs mainly via recombination between insertion sequence elements (ISE), specifically IS*481* (Parkhill *et al*., 2003; Caro *et al*., 2006). IS*481* comprises a 1 kilobase (kb) transposase gene flanked by repeats. The *Bordetella pertussis* genome contains ca. 250 copies of IS*481*, several copies of IS*1002* and ca. 20 copies of IS*1663*. In *B. pertussis* most of the sites of deletions and recombination are flanked by IS elements, evidence for their role in the evolution of the *B. pertussis* genome (Parkhill *et al.*, 2003).

1.5 The genome and genetic diversity of Bordetella pertussis

1.5.1 Genome

Whole genome sequencing (WGS) has made it possible to conduct an in-depth, thorough analysis as a means of enhancing the understanding of the virulence, pathogenicity and evolution of the *Bordetella* species. The first *Bordetella* genome project generated and analysed the genome sequences of a representative strain of *B. pertussis, B. bronchiseptica* and *B. parapertussis* (Parkhill *et al.*, 2003), revealing species-specific genes. *B. bronchiseptica* had more than 600 species-specific genes compared to *B. pertussis* and *B. parapertussis*, possibly reflecting its greater host range as a wider niche range is likely to require greater metabolic capacity and greater ability to respond to differing environments. *B. pertussis* had 114 species-specific genes, and *B. parapertussis* had 50 species-specific genes. However, it was later found that these genes were present in other *B. bronchiseptica* strains, suggesting that *B. pertussis* and *B. parapertussis* do not contain any species-specific genes and have not undergone any gene acquisition during their evolution.

1.5.2 Genetic diversity of B. pertussis

Pre-genomics, the study of genetic diversity and epidemiological typing in *B. pertussis* was based on methods such as multilocus enzyme electrophoresis (MLEE), ribotyping, RAPD IS*1002*-based fingerprinting, variable number of tandem repeats (VNTR) and pulsed field

gel electrophoresis (PFGE) (Mooi *et al.*, 2000; Advani *et al.*, 2013; Van der Zee *et al*., 1997). Studies based on these methods found *B. pertussis* strains to be very highly conserved as well as appearing to have very limited genetic diversity when compared to other bacteria such as *Escherichia coli, Streptococcus pyogenes and Helicobacter pylori* (Go *et al*., 1996). Van der Zee *et al* (1997) conducted multi-locus enzyme electrophoresis (MLEE) on 18 *B. pertussis* strains from the Netherlands, USA and Japan and found the population structure to be largely clonal in nature, even though the strains were isolated from different countries. Advani *et al* (2013) carried out PFGE analysis of 396 strains from a number of European countries where different vaccine programmes were used as a means of determining the epidemiology and spread of *B. Pertussis* over a 10-year period. They found 5 common profiles that were dominant in these countries, suggesting that strains containing 'successful profiles' are able to spread to different countries and induce large shifts in the *B. pertussis* population. Although methods like PFGE and MLEE are useful, they are somewhat insensitive, their capabilities to identify genetic relationships limited (van Gent *et al*., 2011) and they may detect only species diversity as opposed to strain diversity. More recently whole genome sequence (WGS) analysis has become the gold standard for defining the true genetic relationships and diversity among *B. pertussis* strains over a period of time, due to its very high resolution. The Tohama I genome is typically used as a reference genome. Once strains of *B. pertussis* are sequenced, the sequences can be mapped to this reference genome, and single nucleotide polymorphisms (SNPs) identified. SNPs are defined as single base pair changes in the DNA sequence and are the most common form of genetic variation. The SNP within a coding region will either be a synonymous mutation (SM), whereby the mutation does not change the amino acid sequence, also known as a silent mutation, or a nonsynonymous mutation (NSM), where the SNP alters the amino acid sequence, which may or may not alter the protein's characteristics. SNPs found in non-coding regions such as promoter regions can affect the binding capabilities of RNA polymerase and transcription of genes. Using SNP-based methods to identify genetic diversity between strains has been shown to be more effective than the traditional PFGE and MLEE methods, where false homoplasies in phylogenetic trees have been shown (van Gent *et al.*, 2011). Maharjan *et al* (2008) were the first to identify genome-wide SNPs, between an Australian *B. pertussis* strain and Tohama I. They used comparative genomic sequencing and identified 70 SNPs in a 1.4 Mb section of the genome. They calculated the SNP rate of *B. pertussis* to be 1 SNP per 20,000 bp, confirming that the genetic variation in *B. pertussis* is incredibly low. However, this study used only two strains and only one third of the genome was studied. Further research by

Bart *et al* (2010) used 6 Dutch strains, two of which were isolated prior to the introduction of vaccination, and 4 strains isolated in 1999-2000. This identified 471 SNPs. They calculated a SNP density of 1 SNP per 8675 bases. The location of these SNPs highlighted diversifying selection in more recent strains that are circulating in vaccinated populations compared to pre-vaccination strains. Like Mahajan *et al* (2008), they concluded that *B. pertussis* has very limited genetic variation and is a highly monomorphic pathogen.

The most definitive study to date was conducted by Bart and colleagues (2014) that conducted WGS analysis of 343 strains isolated between 1920-2010, from 19 countries, enabling the investigation of genetic variation of isolates worldwide, over time.
This produced a phylogenetic tree based on the 5,414 SNPs found among the strains and identified two distinct lineages that diverged around 2000 years ago. It was concluded that these two branches might reflect two separate introductions of *B. pertussis* in to the human host population. There was very little geographical clustering of strains. An increase in genetic diversity of *B. pertussis* strains was observed following the introduction of the WCV, with more recent strains further diverging from the surviving lineage. It was concluded that certain lineages of *B. pertussis* strains, especially those with a fitness advantage, spread rapidly across the globe. WGS analysis reveals that gene loss is ongoing, due to intragenomic recombination between IS*481* repeats (Caro *et al.* 2006; Bart *et al* 2014).

Recombination between IS*481* elements not only cause deletions but also genome rearrangement (Parkhill *et al*., 2003). Gene rearrangement via insertion sequences plays a major role in the genetic and antigenic diversity of other species such as *E. coli*, where IS-mediated rearrangements are reported to induce genetic variation (Drummelsmith *et al*., 1997). Research by Stibitz and Yang (1997) identified differences in gene order between laboratory-adapted *B. pertussis* strains. Similarly, Park *et al* (2012) also identified considerable genomic rearrangements between Tohama I and another laboratory-adapted strain, 18323. Stibitz and Yang (1999) also found chromosomal rearrangements and inversions in clinical strains isolated from an outbreak in Canada (Stibitz and Yang, 1999). However at the time of the study the mechanisms behind this phenomenon was unknown. Later, research by Brining *et al* (2006) identified that most of the rearrangements were flanked by IS*481* repeats hypothesizing that these IS elements may be responsible for some differences in gene order and transcript abundance between *B. pertussis* strains. It was

concluded that *B. pertussis* used this mechanism to create genetic variation, in a species with very limited variation in gene repertoire.

1.6 The formidable hurdles to controlling pertussis

1.6.1 Resurgence of B. pertussis

The introduction of the pertussis vaccine programme in the UK in 1957 proved extremely effective in reducing rates of pertussis morbidity and mortality. Prior to vaccination, the number of cases reached over 170 000 per year during the 1940s (Fig. 1.1).
However, over the last 20 years, numerous countries that have high pertussis vaccine coverage have suffered a resurgence of pertussis. This includes France (Zepp *et al*., 2011), USA (Rohani and Drake 2011) and Australia (Octavia *et al*., 2012). The Netherlands experienced a large-scale outbreak in 1996, when 2,771 cases were observed, compared to the previous year, in which only 319 cases were reported (de Melker *et al.*, 2000). From 1996, cases of pertussis remained high with another resurgence reported between 2011 and 2012 (van der Maas *et al.*, 2013). Similarly, California has experienced similar patterns with large-scale outbreaks of 9,000 cases in 2010, and again in 2014, with 9935 cases.

1.6.2 The UK 2012 epidemic

Although vaccine coverage has remained ca. 95% in the UK (Health Protection Report, 2015), in 2012, England and Wales experienced a large scale outbreak of pertussis (Fig1.3). A total of 9,711 laboratory-confirmed cases were recorded, leading to fourteen deaths in infants less than 3 months of age (Public Health England, Pertussis notifications and deaths, England and Wales: 1940 – 2014). This was much greater than the previous recent 'peak' year in 2008, in which 902 cases were reported with 6 deaths in infants (Public Health England, 2014). Thus the 2012 resurgence was considered 'real' because there was a marked increase in the number of deaths than what would normally be expected during the cyclic peak years. Specifically, the number of cases began to rise towards the end of 2011 and peaked in October 2012 (Fig.1.3). The highest number of cases was seen in the 15-40 year age group, a group that would have received the WCV. The reason behind the high number of cases in this group is considered due to the lack of boosting by natural exposure to *B. pertussis* strains because vaccine coverage was high during their time of birth (Amirthalingham *et al*., 2013). Although the incidence has declined since the peak of 4621 cases in 2013 and a further reduction to 3388 cases in 2014 (27% decrease) the number of cases still remains much higher than pre-2012 outbreak years.

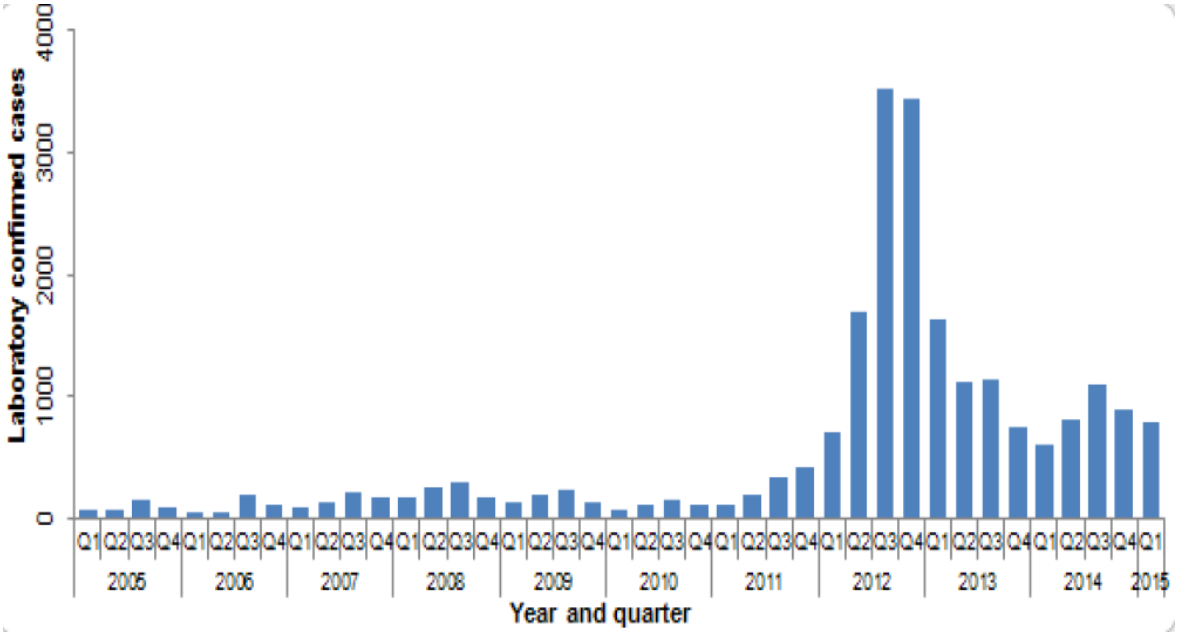

Fig.1.3 The number of laboratory confirmed cases of pertussis in England and Wales from January 2005 to March 2015 (Public Health England. Enhanced pertussis surveillance, 2014).

1.6.3 Changing epidemiology of pertussis resurgence.

In countries experiencing pertussis resurgence, there has been a very noticeable shift in epidemiology (Clark, 2014). Historically, reported disease incidence has been highest in infants. Even after the widespread introduction of vaccination, although the numbers of cases decreased substantially, the 3-5 yearly peaks of disease observed in the prevaccine era remained. This suggests that although there was less disease, transmission of *B. pertussis* was not affected to the same degree. This situation remained until the late 1990's and early 2000's when the reported incidence of disease among adolescents began to increase. In outbreaks in the USA during 2004 and 2005, over 30% of cases were in adolescents (Clark, 2014). A further shift in epidemiology was observed during the USA 2010 outbreak. Although the highest incidence of disease continued to be in the <1 year olds, significant levels of disease were observed among 7-11 year olds (Clark, 2014). In the UK outbreak in 2012, the highest incidence of disease was also in young infants but a large increase over previous years was also recorded in 10-14 year olds (PHE, 2015). These patterns describe a situation in which protective immunity from ACV vaccination appears to be waning earlier (see below), despite continued high levels of full vaccination coverage. The relatively-short lived immunity induced by ACVs is consistent with this epidemiological picture and has been the main focus of discussions around the use of booster vaccinations to halt this increase in disease among school-aged children. A further consideration to understanding the changing epidemiology of pertussis is that of asymptomatic infection.

1.6.4 Asymptomatic infection.

The epidemiology of *B. pertussis* has been largely defined based on the incidence of pertussis among different age groups. Additional studies have considered mild, or atypical disease by detecting *B. pertussis* among people presenting with chronic cough or other respiratory illness. While these have demonstrated that cases of *B. pertussis* infection are classic whooping cough, they do not address the issue of whether *B. pertussis* is present in apparently healthy people, i.e. whether *B. pertussis* can be carried asymptomatically. The recent findings made using the infant baboon model, described below, suggest that asymptomatic transmission occurs readily and given that the level and duration of colonisation of ACV-vaccinated baboons was greater than in animals vaccinated with WCVs (Warfel *et al*., 2014) it seems probable that asymptomatic infection has increased in humans where ACVs are used. Modeling of incidence data also supports the situation in which asymptomatic transmission is a major driver of the resurgence of pertussis in highly vaccinated populations (Althouse and Scarpino, 2015). This study predicts increasing numbers of asymptomatic infections following the switch from the use of WCV to the use of ACVs, and a subsequent increase in disease incidence. In addition, the advent of serology-based diagnosis has greatly increased the ascertainment of cases as blood samples are available from a wide range of cohorts. In all countries for which there are data, the incidence of seropositive samples greatly exceeds the reported incidence of pertussis disease, often by several orders of magnitude, and this was true for all age groups (Barkoff *et al*., 2015). It is not clear what degree of exposure to *B. pertussis* is required for seroconversion to a level counted as positive in these studies but as the incidence of seropositive samples was so much greater than the incidence of reported disease, it is very probable that asymptomatic infection accounted for a large number of positive samples.

1.6.5 Management of the outbreaks

Considering that the UK has a high level of vaccine coverage (Public Health England 2014), the 2012 outbreak has become an area of increasing concern. Strategies have been put in to place to prepare for these outbreaks in the Netherlands such as an accelerated schedule of the vaccination program as well as the administration of an ACV pre-school booster usually given from 3 years 4 months, which appeared to reduce the incidence rate in both the 4-6 year old group and young infants when comparing the pre- and post-implementation periods (van der Maas *et al*., 2013). However these measures had little to no effect on incidence rates in adults and adolescents, (van der Maas *et al*., 2013). Cocooning strategies have been used in California, involving vaccinating mothers and

fathers of new-borns as well as vaccinating pregnant women. Efficient trans-placental transfer of antibodies from mother to babies has been observed (Gall *et al*., 2011; Leuridan *et al.,* 2011) but whether or not these antibodies give direct protection against pertussis had yet to be established. The importance of carrying out the above measures was highlighted by a study in Canada, France, Germany and USA. Wendelboe and colleagues (2007) estimated that 76-83% of cases were from household members, demonstrating the importance of maternal vaccination and cocooning in controlling outbreaks. In response to the national outbreak in the UK vaccination for pregnant women was offered from 1st October 2012, to protect infants from birth by inducing passive protection via the placenta. Antibody titres against the five vaccine components were measured in mothers who were vaccinated during pregnancy compared to those who were not in a case-control study (Ladhani *et al*., 2015). Initial results from case screening suggested that this approach is effective at protecting newborn babies (Amirthalingham *et al.*, 2014) and this was further corroborated during a case-control study in which vaccine efficacy was estimated to be 93% (95% CI: 81-97%) (Dabrera *et al*., 2015).

Due to the common occurrence of pertussis outbreaks across the world, pertussis has been named the most prevalent bacterial vaccine preventable disease worldwide which contributes to the consensus that pertussis is a resurgent disease that may no longer be effectively controlled by current vaccination programmes.

1.7 Explanations for the resurgence

1.7.1 Is resurgence just greater awareness of pertussis disease?

Pertussis infections are reported following clinical diagnosis and/or laboratory confirmation by culture, PCR or serology. PCR and serology are considered more sensitive than traditional culture-based diagnosis. This together with greater awareness of pertussis could be a possible explanation for the increase in the number of cases recorded. However, despite diagnostic methods not changing from 2008-2012 in the UK, there was still a sharp increase in the number of cases in 2012.

1.7.2 The war of the vaccines

Another explanation for the outbreak is waning immunity. Natural infection by *B. pertussis* involves both cell-mediated and humoral responses (Higgs *et al.*, 2012). Upon infection, local macrophages, dendritic cells and neutrophils are the first responders to infection in the respiratory tract (Higgs *et al.*, 2012). Production of interferon gamma (IFN-γ) and IL-

17 further enhances killing of *B. pertussis* by macrophage activation, suggesting that Th1 and Th17 cells play a role in mediating the primary immune response (Higgs *et al.*, 2012). The production of IgG antibodies occurs later during infection and is thought to play a role in complement fixing and opsonizing of bacterial cells to aid in clearance (Higgs *et al.*, 2012).

Determining the efficacy and duration of ACV-induced immunity has become paramount to understanding global outbreaks. As most countries have also used WCVs, comparisons between the efficacies of these two vaccines is possible. Several studies have indicated that vaccination with a whole cell pertussis-containing vaccine is associated with significantly greater protection than vaccination with solely ACVs (Sheridan *et al*., 2012; Witt *et al*., 2013) however outbreaks have still been documented in Countries that have used the WCV, such as Canada (Bentsi-Enchill *et al*). Furthermore, the composition and efficacy of the WCV has been shown to vary as a result of different manufactures and the strains used for the production of the WCVs (Gzyl *et al*., 2004). Several studies indicate that ACV-induced immunity is shorter in duration than WCV- or infection-induced immunity, resulting in those vaccinated with ACV being susceptible to infection at a younger age than those vaccinated with WCV (Rendi-Wagner *et al*., 2006; Warfel and Edwards, 2015; Witt *et al*., 2012). Acosta *et al* (2015) determined vaccine effectiveness in adolescents during the 2012 Washington state epidemic, after individuals completed the 6th pertussis vaccine-containing booster dose. Two to four years post-vaccination, the effectiveness of the vaccine reduced from 73% to 34%.

Qualitative differences in the immunity induced by ACVs compared to WCVs were demonstrated using a recently developed infant baboon model of *B. pertussis* infection and disease (Warfel *et al*., 2012). This model reproduces pertussis symptoms including cough for the study of *B. pertussis* infection biology. However, the ethics and logistics of the use of non-human primates limits study sizes and thus it is not possible to model factors operating at the population level as opposed to the level of the individual host. Animals were immunised with either a 5 component ACV or WCV at 2, 3 and 4 months of age and then at 7 months of age were intranasally challenged with *B. pertussis* (Warfel *et al.,* 2014). Equal numbers of ACV-vaccinated baboons received either 3-component or 5-component pertussis vaccines and the results were pooled. The baboons then underwent nasopharyngeal washes to quantify colonisation, in addition to monitoring of their symptoms. The baboons vaccinated with ACV did not experience severe symptoms of

whooping cough, however, upon challenge, they failed to prevent colonisation by *B. pertussis* and were able to transmit the disease to other baboons. Baboons vaccinated with the WCV had faster clearance of *B. pertussis* compared to those immunised with ACV. The T cell immunity of these baboons was also monitored and WCV-induced immunity mirrored that of a natural infection, demonstrating induction of *B. pertussis* specific T-helper (Th) 17 memory cells and Th1 cells. Baboons vaccinated with ACV mounted a Th1 and Th-2 response, but no Th17 response, which is important for successful clearance. Thus, increasing evidence points to the failure of the ACV to sustain long-lasting immunity against pertussis as a key contributor to resurgence however not all Countries using the ACV have incurred a resurgence (Domenech *et al*., 2016). The WHO SAGE Working Group examined pertussis data from 19 countries and concluded that only 5 out of 19 countries (Australia, Chile, Portugal, USA and UK) supported the presence of a true resurgence. Chile was the only country that used WCV. Changes in other countries could not be separated from changes in surveillance systems and normal epidemic cycles. (WHO SAGE Working group report, 2014)

1.7.3 Is B. pertussis *evolving to evade vaccine induced immunity?*

The hypothesis that *B. pertussis* strains are evolving to evade vaccine-induced immunity has received a lot of attention. The ACVs comprise from 1-5 antigens and thus all selection pressure arising from vaccine-mediated immunity is targeted on these proteins and would likely select for mutations in their genes. The allelic profile of the five vaccine antigen encoding genes in the vaccine strain Tohama I is *ptxA2-ptxP1, prnA1, fim 3-1, fim 2-1* (Litt *et al*., 2009; van Loo *et al*., 2002) whereas many currently circulating strains harbour a distinct allele profile (*ptxA1-ptxP3, prnA2, fim 3-2, fim 2-1*), consistent with an increase in the selective pressures induced on these five antigens and subsequent allelic changes since the introduction of the ACV (Bottero *et al*., 2012; Elomaa *et al*., 2007; Komatsu *et al*., 2010; Mooi, 2010). The expansion of these alleles in the population has been associated with the resurgence of whooping cough in the Netherlands, for example (Mooi *et al.,* 1998). However these allele types have been dominant and circulating in the UK population since the 1990s (Fry *et al*., 2001; Litt *et al*., 2009; Packard *et al*., 2004). The non-vaccine types *ptxA1* and *prn2* have been circulating in the UK since 1984. Thus, resurgence cannot be simply due to the emergence of variants displaying mismatches to the vaccine strains.

Variation in the *ptx* promoter (*ptxP*) has been identified as contributing to the resurgence in various countries. The *ptxP* is 266 base pairs (bp) in length and lies directly upstream of the start codon of the *ptxA* gene. The *ptxP* allele in the vaccine strains is allele type 1 (*ptxP1*) however up to 14 different alleles have been reported (Bart *et al.*, 2014). In particular, the *ptxP* type 3 (*ptxP3*) has largely replaced other allele types and is by far the most dominant allele currently circulating worldwide (Kallonen *et al*., 2011). *PtxP3* is distinguished by a single base pair (bp) change from a guanine (G) to an adenine (A) 90 bp from the start codon of *ptxA*. The *ptxP3* allele is associated with an increase in toxin production as more toxin has been shown to be produced in broth compared to *ptxP1* strains, and thus linked to the resurgence of pertussis (Mooi *et al*., 2009; Lam *et al*., 2012). The genetic background of *ptxP3* strains also appears to play a role in fitness. King *et al* (2013) demonstrated that *ptxP3* strains colonized mice to higher levels than *ptxP1* strains, but also, the genetic background of the *ptxP3* strains improved overall fitness of the strains by increasing expression of other virulence genes suggesting *ptxP3* has both a direct effect but is also a marker for other genetic changes influencing fitness (King *et al*., 2013). Advani *et al* (2011) studied the allele types of *B. pertussis* strains across two regions of Sweden using different vaccines. During 1997-2006, the Gothenburg area, which used the mono-component (PT only) and the 2-component vaccine (PT and FHA) reported cases of whooping cough that were four times higher than other areas of Sweden. A switch from *ptxP1* to *ptxP3* was identified in strains from the Gothenburg area, and was suggested as a possible reason behind the increase in the number of cases in this area. It was concluded that the difference in cases seen between the two Swedish areas could be explained by use of the mono-component vaccine in the Gothenburg area possibly accelerating the evolution of strains containing the *ptxP3* allele, providing strong selection for strains producing increased amounts of toxin that might overcome solely toxin-focused immunity. The *ptxP3* allele is now the dominant allele circulating worldwide and it is believed this change in allele type is in response to vaccine-mediated pressure, with an increase in pertussis notifications associated with this switch (Mooi *et al*., 2009; Octavia *et al*., 2012; van Gent *et al.*, 2012). This allelic change may have a fitness advantage in the current *B. pertussis* population due to selection presssure. The selective advantage of this allele type over other alleles such as *ptxP1* has consequently enhanced its spread through the population. The study by Bart *et al* (2014) enabled a detailed study of effects of vaccination on population structure using strains from a 19 Countries. Current outbreak strains have expanded from a lineage that arose prior to vaccination and have diversified since. This was somewhat surprising as it was assumed that the introduction of the vaccine would

reduce genetic diversity, eradicating many clones with only those sufficiently mismatched to the vaccine strains surviving. Using Bayesian methods it was estimated that the *ptxP3* allele arose once during the WCV period, between 1974-1977 and subsequently spread worldwide. The *fim2-2* allele arose during the WCV period and the WCV/ACV period, however the vaccine type *fim2-1* allele has remained the dominant type worldwide. In contrast, the non-vaccine type *fim3-2* has risen in frequency from 1% during the WCV era, to 37% in the ACV-era suggestive of a fitness advantage to strains carrying this non-vaccine allele in the ACV-era (Bart *et al.*, 2014). Strains harbouring the *ptxA1* allele increased in frequency from 1% frequency during the WCV period, to 90% in the ACV period. Thus, while it is likely that this allele arose prior to the introduction of vaccination, it is possible that the use of vaccines imposes a selective advantage on *ptxA1* strains. Bart *et al* (2014) concluded that due to the lack of geographical clustering of *B. pertussis* strains, there is a rapid global spread of *B. pertussis* strains that appear to be evolving in response to vaccine driven immune selection, and thus, vaccine evasion. This analysis highlights the large spread of *B. pertussis* strains at a global scale. It identifies that transmission of *B. pertussis* is not limited within one country and that those alleles that are dominant are so worldwide. The switch in allele type and successful spread in the population could possibly be in response to vaccine-induced immunity and it is hypothesized that these vaccine antigens are evolving to evade vaccine-induced immunity across the world.

Much of the attention regarding vaccine-driven evolution of *B. pertussis*, and the most compelling evidence for it, stems from variation in *prn*. Prn contains two immunodominant repeat regions (Boursaux-Eude and Guiso, 2000), which are comprised of a glycine-glycine-X-X-proline sequence and a proline-glutamine-proline repeat region, designated region 1 and region 2, respectively. These repeat regions have been suggested to create a single discontinuous epitope recognised by vaccine antibodies (Hijnen *et al.*, 2007). The *prn* gene also encodes a tripeptide arginine-glycine-aspartic acid or RGD cell binding site motif, located directly before the first repeat region in the *prn* gene. This RGD motif mediates cell attachment to a number of mammalian cells (Ruoslahti *et al.*, 1996). Further analysis of this motif suggested it also plays a role in the attachment to mammalian cells via integrins involved in uptake, as Prn-negative strains were observed to have a 30-40% reduction in attachment to Chinese hamster ovary cells compared to Prn-expressing strains (Leininger *et al*, 1992, 1991).

Prn is thought to be a significant component of the acellular pertussis vaccine as the 3-component vaccine containing Prn, FHA and PT was found to be considerably more efficacious than the 2-component vaccine containing just PT and FHA in a clinical study (Hewlett *et al.*, 1997). The correlations between levels of antibodies to acellular vaccine components and protection against pertussis disease were studied after household exposure to *B. pertussis* in clinical trials in Sweden. The correlation between the levels of anti-pertactin antibodies and antibodies against copurified Fim2/3 antigen and disease was clear and statistically significant (Storsaeter et al. 1998). Similar results were also reported by Cherry *et al* (1998) in a German household study. Furthermore, only anti-Prn antibodies, and not anti-PT, anti-FHA or anti-Fim2/3 antibodies, aid in phagocytosis of *B. pertussis,* providing a biological explanation for the correlation between protection and level of anti-Prn titres (Hellwig *et al.*, 2003). A global collections of strains harboured 11 distinct *prn* alleles, which were characterised by polymorphisms in repeat regions (King *et al.*, 2001), with the WCV era being dominated by the vaccine type *prn1*, which was eventually replaced by the *prn2* and *prn3* alleles, with *prn2* dominating during the ACV period (65%). Anti-Prn1 antibodies do not bind to Prn2 or Prn3 as effectively, and monoclonal antibodies (mAb) directed against the epitope which harbours the polymorphisms are type-specific (van Gent *et al.*, 2011; King *et al.*, 2001). This suggests that variation in Prn does affect recognition by vaccine-induced immunity.

Of note, recently, a growing number of Prn-deficient *B. pertussis* strains are being isolated. Prn-deficiency arises from different mechanisms. Insertion of IS*481* or IS*1001* into *prn*, an 84bp deletion at the start of the coding sequence, as well as a mutation at position 1479 resulting in a stop codon have been reported (Otsuka *et al*, 2012; Lam *et al*, 2014; Hegerele *et al*, 2012).

The prevalence of Prn-deficient strains varies between countries (Pawloski *et al.*, 2014). Countries including Australia, Finland, France, Japan, and the USA have reported Prn-deficient strains (Lam *et al*, 2014; Barkoff *et al*, 2012; Bouchez *et al*., 2009; Otsuka *et al*, 2012; Pawloski *et al*., 2014). Zeddeman and colleagues (2014) investigated the frequency of Prn-deficiency in 261 *B. pertussis* strains from 6 European countries. Prn deficiency arose several times independently, and the first countries to experience Prn deficient strains introduced the ACV much earlier than other countries where Prn-deficient strains are now starting to emerge. Interestingly, Denmark has not reported any Prn-deficient strains. This country uses the mono-component ACV, which contains PT only. In the USA, Pawloski *et*

al (2014) investigated the frequency of Prn-deficient strains before and during the 2010 California outbreak and the Washington State outbreak in 2012 and found the frequency of these strains had dramatically increased, with more than 50% of the strains being Prn-deficient in 2012. Furthermore, Martin *et al* (2015) carried out a retrospective study examining the relationship between Prn-expression by strains, vaccine history and clinical presentation in patients. This showed that vaccinated individuals in the USA were more likely to have been infected with a Prn-deficient strain than a Prn-expressing strain.

These data suggest that the introduction of the ACV may have exerted pressure on *prn* to evolve in order for strains to evade ACV-induced immunity. As Prn has been shown to induce a protective immunity, possibly through high antibody titres, as demonstrated by vaccination studies (Higgs *et al.*, 2012), the loss of this antigen could improve evasion from the host immune system in strains circulating in highly vaccinated human populations. Using the mouse model of *B. pertussis* infection it was demonstrated that naïve mice were colonized to the same degree by Prn-expressing or Prn-deficient strains. However, in mice immunized with ACV and then subsequently challenged with *B. pertussis*, Prn-expressing strains were cleared more quickly than Prn-deficient ones (Hegerle *et al*., 2014). Finally, a very recent study developed this further and assessed direct competition between Prn-expressing and Prn-deficient strains in the murine infection model (Safarchi *et al*., 2015). Coinfection of naïve mice and ACV-immunised mice with equal numbers of a Prn-expressing and a Prn-deficient strain revealed dramatic differences. In naïve mice the Prn-expressing strain became dominant by day 3 post-inoculation and accounted for over 90% of the *B. pertussis* recovered by day 14, demonstrating a distinct fitness advantage from expression of Prn in these hosts. However, in immunized mice, the reverse was observed, as the Prn-deficient strain accounted for over 90% of recovered bacteria by day 14 (Safarchi *et al*., 2015). Together these data strongly suggest that the loss of expression of Prn generates a fitness advantage to *B. pertussis*, but only in hosts immunized with an ACV.

In summary, pertussis vaccines have proved highly successful in reducing the burden of *B. pertussis* and remain the best method of control. However, due to the introduction of the ACVs, vaccine-mediated immune pressure is now focused on these five vaccine antigens in countries using these vaccines. A worldwide resurgence of pertussis has been linked to switch to the use of ACV and the evolution of *B. pertussis* away from vaccine-mediated immunity. The fact that Prn deficient strains are increasing is good evidence to support the

hypothesis that strains are evolving to evade vaccine-induced immunity. However, resurgence is also associated with a rise in incidence in older age groups that were vaccinated using WCVs. This might suggest that adults have had greater exposure to *B. pertussis* in recent years or this could be further evidence for recent strains being able to evade vaccine-mediated immunity. Further analysis of the UK 2012 pertussis outbreak will increase our understanding of the evolution and phenotypic behaviour of *B. pertussis* in the face of vaccine-induced immunity and help to provide reasons for pertussis resurgence.

2

Aims and Objectives

Between 2011 and 2012, the United Kingdom experienced a large-scale epidemic of whooping cough. Therefore, the aim was to investigate whether or not the 2012 outbreak strains are evolving to evade vaccine induced immunity.
In order to fully understand the reasoning behind this resurgence, identifying the evolutionary strategies and behaviours of *B. pertussis* is paramount.

Whole genome sequencing was performed on 100 clinical strains of *B. pertussis* in order to investigate genetic differences between outbreak and pre-outbreak strains. The specific questions were:

- Do the 2012 outbreak *B. pertussis* strains form a single dominant clone that contributed to the resurgence?

- Do the outbreak strains have an allelic profile that is specific to this group and could have contributed to the 2012 outbreak?

- Are the vaccine antigen genes evolving at a faster rate than those coding for other surface localised proteins?

The level of Prn expression was investigated in a panel of pre-outbreak and outbreak strains. The specific question was:

- Do the outbreak strains express a significantly different level of Prn that may have provided a fitness advantage in a highly vaccinated population?

The same set of strains was also used to investigate differences in recognition by post-vaccine sera. The specific question was:

- Are outbreak strains recognised at a significantly lower level than pre-outbreak strains?

3

Materials and Methods

3.1 Bacterial strains

The genome of *Bordetella pertussis* Tohama I was determined by Parkhill *et al* (2003) (EMBL accession number BX470248) and was used as the reference genome in this study. 100 *B. pertusiss* isolates were obtained from the National Reference Laboratory, Respiratory and Vaccine Preventable Bacteria Reference Unit at Public Health England (Appendix A). Five strains were collected between 1920-1956 (defined as the 'pre-vaccine' era), six strains collected between 1957-2000 (WCV era) and 89 strains were collected between 2000-2012 (ACV era). Serotyping was performed using sera specific for antigens 1, 2, and 3 (89/596, 89/598, and 89/600, respectively; National Institute for Biological Standards and Controls, Potters Bar, United Kingdom) as previously described (Litt *et al*., 2009). Tohama I (accession number BX470248), isolated in Japan in 1954, is widely studied and provides the reference genome sequence of *B. pertussis* (Parkhill *et al.,* 2003). Tohama I is also used to manufacture Infanrix and Boostrix vaccines which are used in the UK.

The genome sequence data of these strains have been deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena/).

3.2 Growth Conditions

3.2.1 B. pertussis

B. pertussis strains were stored at -80°C in PBS/20% glycerol stocks until required. *B. pertussis* was streaked on to charcoal agar (Oxoid, UK) plates and placed in a 37°C incubator for 72 hours. For broth cultures, *B. pertussis* was harvested from the plate, resuspended in 2ml PBS (Oxoid, UK) and the OD_{600} measured. *B. pertussis* was grown in modified Stainer Scholte (SS) broth made at either Bath University (Table 3.1.a) with added supplements (Table 3.1.b) or Public Health England, Porton Down (Imaizumi *et al.*, 1983) (Table 3.1.c & d). Cultures were inoculated to a starting OD_{600} of 0.1 and incubated at 37°C, with shaking at 180rpm.

Table 3.1a Components of Stainer Scholte (SS) broth prepared at Bath. Components were dissolved in 90% of the final volume, adjusted to pH 6.7, brought to the final volume and autoclaved.

Final concentration	Chemical	Amount to add to 1L (g)
	Casamino acids	10
57mM	L-glutamic acid	10.7
2.1mM	L-proline	0.24
43mM	NaCl	2.5
31mM	KH_2PO_4	0.5
2.7mM	KCl	0.2
0.49mM	$MgCl_2.6H_2O$	0.1
0.18mM	$CaCl_2.2H_2O$	0.0265
50mM	Tris Base	6.1
	Heptakis (2,6-di-*O*-methyl)-β-cyclodextrin (Sigma)	1

Table 3.1b Components of Supplements (100x) added to SS broth immediately prior to use.

Final concentration (in 1x)	Chemical	Amount to add to 10ml (g)
0.33mM	L-Cysteine	0.04
36μM	$FeSO_4.7H_2O$	0.01
33μM	Niacin	0.004
0.49mM	Glutathione	0.15
2.3mM	Ascorbic Acid	0.4

Table 3.1 c. Components of SS broth made at PHE, Porton Down. Solution B, C and D are to be made separately then added to the final SS media (Table 3.2d).

Solution B

Ingredients	**Amount for 1 L**	**Unit**
L-Cystine	12.5	g
Concentrated HCl	222.5	ml
Milli Q Water	777.5	ml

Solution C

Ingredients	**Amount for 1L**	**Unit**
$FeSO_4.7H_2O$	2.5	g
Milli Q Water	1000	ml

Solution E

Ingredients	**Amount for 1L**	**Unit**
Nicotinic Acid	1	g
Milli Q water	10000	ml

Table 3.1.d SS broth The first 12 (solid) ingredients were added to half total volume of water and stir to dissolve. Solutions B, C and E were added and then made up to the final volume. The pH was adjusted to 7.6 and the conductivity checked. The solution was then sterilsed by filtration.

Ingredients	**Amount for 1L**
L-glutamic acid sodium salt	10.7g
NaCl	2.5g
KH_2PO_4	0.5g
$MgCl_2.6H_20$	0.1g
$CaCl_2.2H_2O$	0.026g
Tris Buffer	6.1g
KCl	0.2g
L-Proline	0.24g
Glutathione	0.15g
Ascorbic Acid	0.4g
Difco casamino aicds	10g
2,6 O-dimethyl beta cyclodextrin (Teijin Ltd Japan)	1g
Solution B	4ml
Solution C	4ml
Solution E	4ml
Milli Q water to	1000ml

3.3 Genetic analysis of UK *Bordetella pertussis* strains

3.3.1 DNA Preparation

Genomic DNA extraction was performed using the Qiagen DNeasy Blood and Tissue kit according to the manufacturer's instructions.

3.3.2 DNA Sequencing and Single Nucleotide Polymorphism (SNP) Identification

Twenty four isolates were sequenced previously (Bart *et al*., 2014). For the remainder, multiplex libraries, with fragment sizes between 300 and 500bp, were prepared as previously described (Harris *et al*., 2010) with modifications (Quail *et al*., 2012) and sent to the Sanger Institute for Illumina sequencing in October 2012. Reads for each isolate were aligned to the Tohama I reference genome using SMALT version 0.7.4 (http://www.sanger.ac.uk/resources/software/smalt/). Base calls were made as previously described (Harris *et al*., 2010), using a combination of samtools, mpileup and bcftools (Danecek *et al*., 2011), allowing SNPs, and small insertions and deletions relative to Tohama I to be identified. Five strains produced poor quality sequence and were excluded from the analysis, resulting in 95 strains being taken forward for analysis.

3.3.3 Phylogenetic Analysis

Maximum likelihood phylogenetic analysis was carried out on variable sites from across the whole genomes using RAxML under a GTR evolutionary model and a gamma correction for among site rate heterogeneity (Stamatakis *et al*., 2006). 100 random bootstrap replicates were run to provide support for relationships identified in the tree. SNPs were reconstructed on to the phylogenetic tree using parsimony.

3.3.4 Analysis of SNP Densities

SNP densities (SNP/bp) within vaccine antigen genes (9 genes: *fhaB*, *prn*, *fim2*, *fim3*, *ptxA-E*) or 'cell surface' functional category genes (591 genes, as categorised previously (Parkhill *et al*., 2003)) were calculated by counting the number of SNPs per bp of each gene. The difference between the mean per gene SNP densities of vaccine antigen genes and cell surface genes was calculated. The significance of this difference was calculated using a non-parametric Monte Carlo simulation. In our randomizations of all the data, preserving relative sample sizes, it was observed how often a difference as large, or greater than the difference above was found, by repeated randomly resampling two samples of the same size as above. Using this protocol, if n is the number of observations that have

greater than or equal to the observed difference in SNP density and *m* is the number of simulations (in this case, 10 000), then $P = (n+1)/(m+1)$ is the unbiased estimator.

A similar procedure was used to compare SNP densities in vaccine antigen genes between eras. To account for differences in SNP densities between strains from the different eras, the SNP densities of the vaccine antigen genes were normalised by the SNP densities of all the genes considered (vaccine antigen and surface protein encoding genes). A non-parametric Monte Carlo simulation compared the normalised SNP densities in the ACV antigen genes in ACV-era strains with pre-ACV era strains, with *P* determined as above.

3.3.5 Allele Typing

The different alleles of *prn*, *ptxA*, *ptxP*, *fim3* and *fim2* genes have been previously described (Mooi *et al*., 2010) and were used to identify allele types from DNA sequence.

3.3.6 Analysis of prn from UK50

The *prn* locus was amplified from UK50 by PCR using primers 5′-CCGCTGATTCGCCACAAG-3′ and 5′-GTGCGGTACTTGCCCTTG-3′. Primers were designed flanking the Prn gene sequence, ensuring that the full *prn* sequence was amplified. This sequence was copied from the Tohama reference genome into a Microsoft Word document. Potential primer sequences were identified by eye and the presence of SNPs in these regions were checked against UK50 to ensure efficient binding. The Biolab TM calculator (http://tmcalculator.neb.com/#!/) was used to identify appropriate annealing temperatures as well as the GC content of the forward and reverse primers. PCR products were cloned using the Gateway system (Invitrogen, Paisley, U.K.) and sequenced by Eurofins Genomics (Ebersberg, Germany) utilising standard M13 forward and reverse primers and internal primers 5′-GCGCACGCCTGTCCAAAG-3′ and 5′-TAGCGAGCCAGCACGTAG-3′.

3.3.7 Analysis of Differences in DNA Content Among Strains

To detect gene loss from strains, compared to the DNA content of Tohama I, coverage plots generated using the paired end reads mapped to this reference genome were used to create a heat map. DNA sequence contigs that did not map to Tohama I were analysed using Blastn and Blastx (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.4 Detection of pertactin expression in clinical isolates of *B. pertussis*

3.4.1 Formaldehyde killed B. pertussis *strains*

Due to the aerosol infection risk of analysis of live *B. pertussis* by flow cytometry, bacteria fixed by incubation in 2% formaldehyde for 1h were analysed. This protocol has previously been used for analysis of Fim expression (Vaughan *et al*., 2014). *B. pertussis* was grown on charcoal plates and then grown in 50ml SS Porton broth. The 50ml culture was then split in to two 25ml Falcon tubes (VWR), pelleted by centrifugation at 3000xg for 15 minutes and the supernatant discarded. Pellets were resuspended in 25ml 2% formaldehyde and placed in a 37°C shaking incubator (180rpm) for one hour. Cells were pelleted by centrifugation at 3000xg for 15 minutes, the formaldehyde discarded, and the cells washed by resuspending in PBS. Samples were centrifuged as above and re-suspended in 10ml PBS. 100µl of the sample was inoculated onto agar plates and incubated at 37°C for 5 days to ensure all cells had been killed. The sample was then divided into aliquots and stored at -20°C. It is understood that formaldehyde fixation may alter protein structure to some degree. Although this has not been confirmed for Prn, all samples received the same fixation treatment allowing quantitative differences between them to be observed.

3.4.2 Binding of anti-Prn Mab to B. pertussis *measured by flow cytometry*

In order to compare expression of Prn on the surface of *B. pertussis*, a 96 well plate assay was performed. Two microlitres of anti-Prn monoclonal antibody, Pem#80 dated 3/11/2010, 1.8mg/ml (a gift from Guy Berbers RIVM) was pipetted in to the designated wells of a 96 well plate. 198µl of formaldehyde-killed *B. pertussis* were then transferred to the wells at an OD_{600} of 1, which was done in duplicates for each strain. Plates were incubated at 25°C for 30 minutes with shaking at 190rpm, to allow binding of the monoclonal antibodies to Prn. Plates were centrifuged at 3060xg for 5 minutes to pellet the killed bacteria and bound antibodies. Goat FITC-conjugated anti-mouse IgG secondary antibody (Jackson Immunochemicals, 115-096-062) was added to the wells at a concentration of 1/500 and incubated for 20 minutes at 4°C to allow binding of the secondary antibodies to the anti-Prn monoclonal antibodies. Duplicate wells containing pelleted bacteria and secondary conjugate only were used as fluorescent background controls (C) to quantify non-specific binding of the secondary antibody.

The plate was centrifuged at 3060xg for 5 minutes, the bacteria washed using 200µl of 2.5% bovine serum albumin (BSA) in Hanks Balanced Salt Solution (HBSS) and then centrifuged again. The pellets were resuspended in 200µl of 2.5% BSA in HBSS.
The fluorescence (FI) of each sample was read in the plates using the Beckman Coulter CyanADP flow cytometer at PHE Porton. The software used for analysis was Summit 4.3 (Beckman Coulter, UK). The C value produced from the conjugate-only control wells was subtracted from the FI readings of the test wells (FI-C).

3.4.3 Binding of post ACV IgG to B. pertussis *determined by flow cytometry*

The procedure was the same as that above, however 2µl of post-vaccine sera (Table 3.4) were added to the wells instead of anti-Prn Mab. Throughout the assays a range of different post-vaccine sera from different individuals was used due to the limited volumes available. Anti-human FITC conjugated antibody (Jacksons Immunochemicals, 109-096-088) was used as secondary antibody.

Table 3.4 Post vaccine sera and the date of extraction

ID	**Time post Repevax (ACV5) vaccination**
POST 4	1 month
POST 7	5 months
POST 9	7 month
POST 13	1 month
POST 14	1 month
POST 2464	1 month
POST 2669	1 month

3.4.4 Statistical analysis

Comparison of Prn expression and post-Repevax recognition between historical (1920-2011) and outbreak (2012) strains and *ptxP1* and *ptxP3* strains used an unpaired, two tailed t-test, assuming equal variance. Correlation analysis (r) was evaluated using a Pearson's product moment correlation coefficient. The P values were determined using a table of critical values for r (http://www.radford.edu/~jaspelme/statsbook/Chapter%20files/Table_of_Critical_Values_for_r.pdf). The coefficient of variation (Reed *et al*., 2002) (SD ÷ FI-C x 100) was considered acceptable at <35%.

4

Results

Genomic analysis of isolates from the UK 2012 pertussis outbreak reveals that vaccine antigen genes are unusually fast evolving

4.1 Rationale

The resurgence of pertussis disease has been linked to increased surveillance, better diagnostic techniques and switching from the use of WCVs to ACVs. Also it has been proposed that vaccine escape mutants are arising, as ACV-induced immunity is focused on just a few antigens, and changes in these antigens might result in strains that are less well recognised by this immunity (Poolman *et al*., 2014).

The frequency of different alleles of vaccine antigen genes among strains has changed over time (Bottero *et al*., 2012; Elomaa *et al*., 2007; Komatsu *et al* 2010; Mooi *et al*., 2010). The study of genetic changes in *B. pertussis* over time has been hindered by the high levels of homogeneity among *B. pertussis* and the lack of fine-resolution tools. Thus recently the genome sequences of a large panel of *B. pertussis* strains collected from around the world and across many decades were generated and analysed (Bart *et al*., 2014). This provided detailed information about the population structure and evolution of *B. pertussis* revealing significant genetic changes among strains over the last 50 years. However, this panel of strains did not contain isolates collected more recently than 2008, except for 3 isolates from the Netherlands collected in 2009 and 2010, and did not intensively sample a specific outbreak meaning that the genetic make-up of such events is largely unknown.

Here 95 UK strains have been analysed with a focus on strains from the recent UK outbreak. *B. pertussis* strains were isolated from a number of locations across the UK, providing a sample of isolates with a relatively widespread geographical distribution (Fig 4.1a). The age of the patients that strains were isolated from varied however the majority of strains were isolated from young infants (Fig 4.1b). This is proportional to the number of isolates available for each age group, as the majority are obtained from hospitalised infants. Sixty-one of the strains were isolated during 2012 and contained within the analysis, representing the most intensive genomic analysis of a single outbreak to date.

This will extend the previous analysis of Bart *et al* (2014). The aim was to understand the clonal structure of the outbreak and determine if there was evidence for vaccine-mediated immunity driving the evolution of these strains.

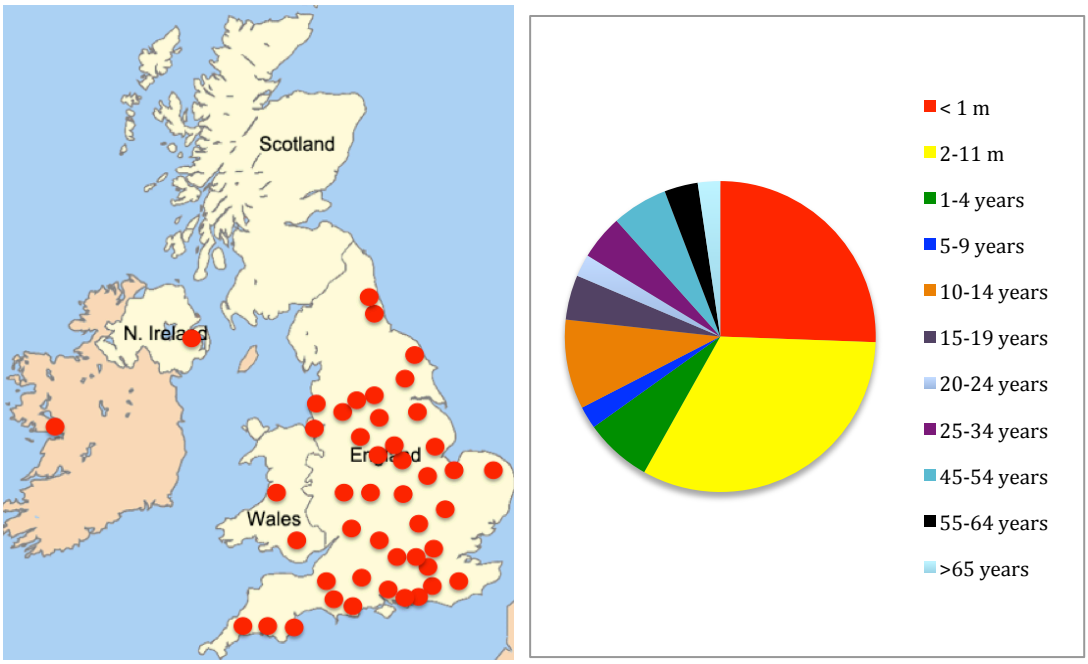

Fig 4.1(a) Illustrates the geographical location of the isolation of UK *B. pertussis* strains used in this study as well as the age group of the patients from which they were isolated **(b)**.

4.2 Phylogeny of UK Strains from 1920-2012

Phylogenetic analysis based on SNPs across the whole genome sequences was performed to understand the phylogenetic relationships between the UK strains (Fig. 4.2). Strains isolated during 1920-1982 form a cluster and are generally distinct from strains from 2008-2012. The most distinct clustering separates strains carrying the *ptxP1* allele from those carrying the *ptxP3* allele, which, as found elsewhere, is the predominant *ptxP* type among recent strains. A further more detailed phylogenetic tree was constructed showing the number of SNP differences between clusters of strains (Fig. 4.3). This phylogenetic analysis was extended to place the UK strains in the global phylogenetic tree described elsewhere (please refer to Bart *et al*., 2014, Fig. 2 and supplementary text sd1, XLSX for the details of the global strains included in this phylogenetic analysis). This reveals that the UK *ptxP3* strains separate into two clusters, distinguished by the presence of the *fim3-2* allele. The UK outbreak strains largely cluster with strains isolated mainly during the early 2000s from a variety of geographical areas including North America, Europe and Australia.

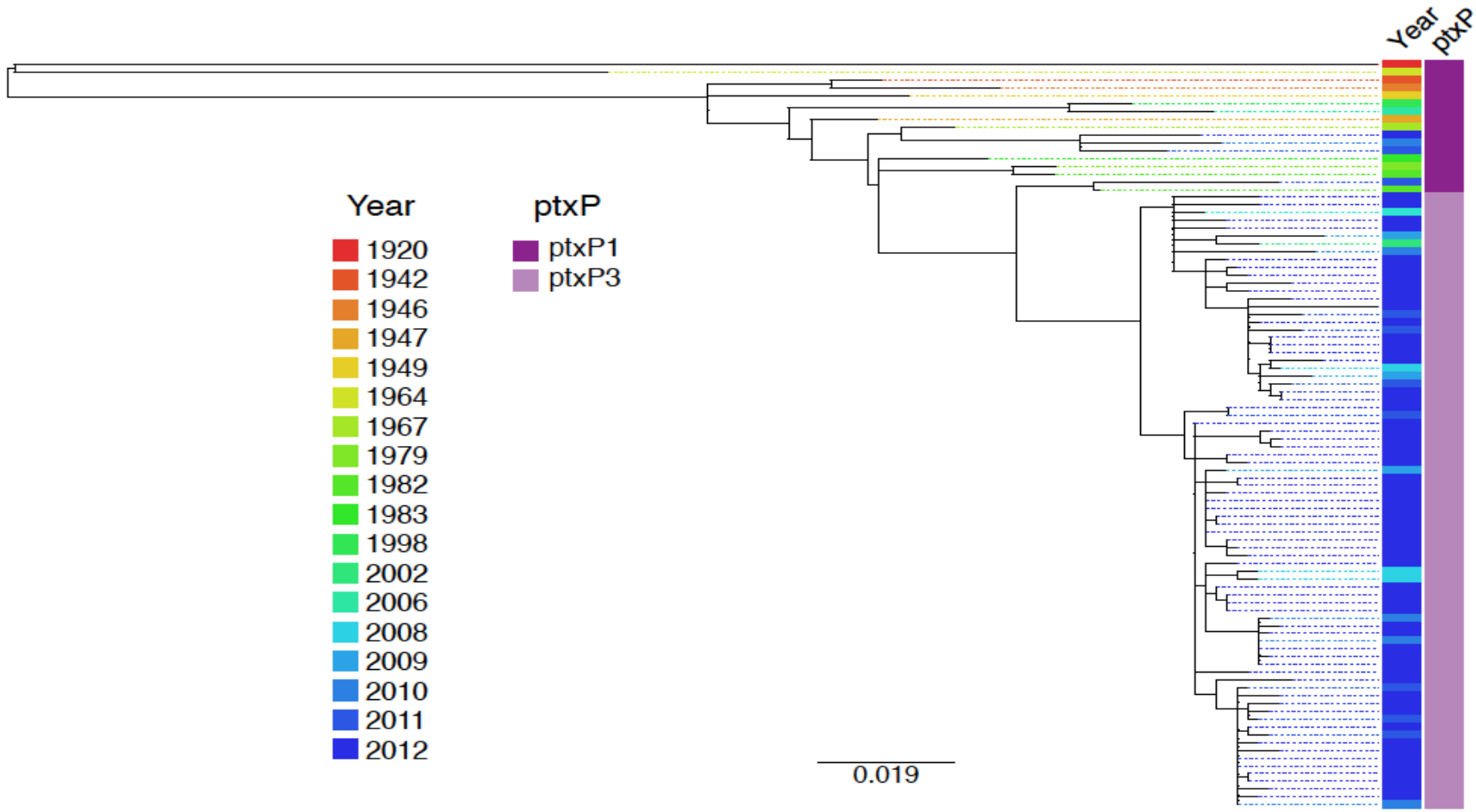

Fig.4.2 Phylogenetic tree depicting the evolutionary relationships among the UK *B. pertussis* isolates studied. Maximum likelihood phylogenetic analysis was performed on variable sites from across the whole genomes, using RAxML. Strains are shaded according to their year of isolation and *ptxP* type.

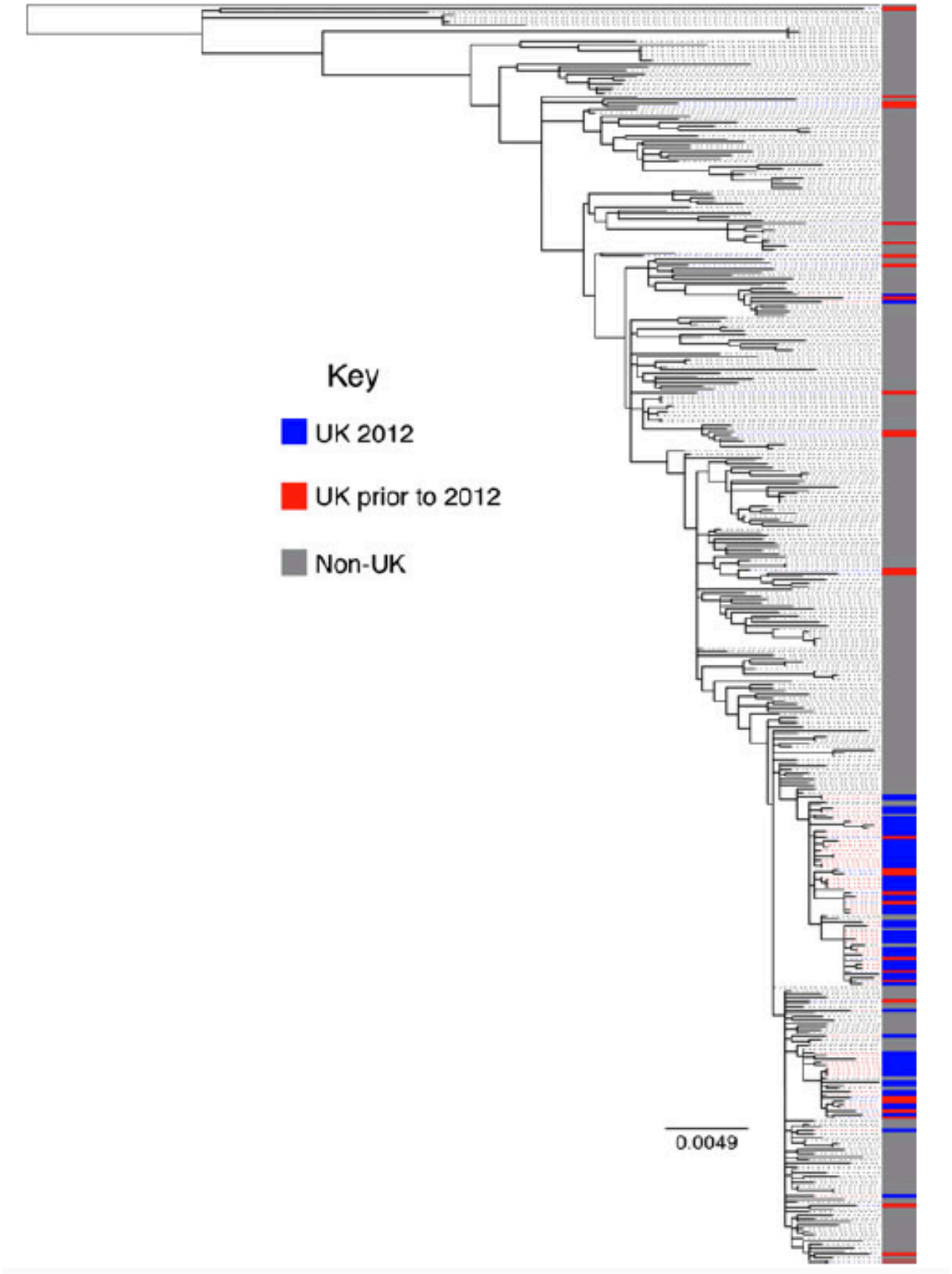

Fig 4.3 Phylogenetic relationships of UK strains within a global context. The UK isolated analysed here are indicated.

4.3 Vaccine antigen allele profiles

Previously, *ptxP3-ptxA1-prn2-fim*3-2 was defined as the dominant allele type circulating in the UK and other countries (Kallonen *et al*., 2009). Typing of alleles among the outbreak

strains reveal no recent change in this profile (Table 4.1). Numerous isolates deficient for the production of Prn have been reported in other countries, and a number of different mutations in *prn* responsible for this phenotype have been identified (Bouchez *et al*., 2009; Lam *et al*., 2014; Otsuka *et al*., 2012). It has been suggested that loss of Prn expression has been selected by vaccine-mediated immune pressure. Interestingly, just a single UK strain, UK50, was mutated for *prn*. This was identified by a lack of sequence reads mapping to a region of *prn*. The *prn* locus was amplified by PCR from this strain and the resulting product sequenced using Sanger sequencing. This identified that a recombination event between two copies of IS*1663* has resulted in a deletion/insertion mutation in which the 5′ 1326 bp of the *prn* coding sequence has been deleted. Aberrant mapping was not observed for any other UK strain. In other countries, a common *prn* mutation arose from insertion of IS*481* into *prn*. Paired end reads were identified in which one read mapped within IS*481* but the other did not and thus derived from the region flanking IS*481*. Mapping these reads to the reference genome identified the position of the copies of IS*481* within each query strain. No IS*481* insertions into *prn* were identified among UK strains. This suggests that few Prn-deficient strains were involved in the UK outbreak. A small sample size, or skewed sample, will affect the number of Prn-deficient strains observed. However the strains analysed here represent strains isolated from numerous regions across the UK and from a number of different age groups. In other countries the frequency of Prn-deficient strains has reached more than 50% (Martin *et al*., 2015). It is clear that in the UK, Prn-deficiency is at a much lower level.

Table 4.1. Frequency (% of strains tested) of vaccine antigen encoding gene alleles among UK strains.

	Period		
	Prevaccine 1920-1956	**WCV 1957-2000**	**ACV 2001-2012**
No. of strains	5	6	84
ptxP 1	100	100	6
3	0	0	94
ptxA 1	20	100	100
2	80	0	0
***Prn 1**	100	84	5
2	0	0	91
3	0	16	3
4	0	0	1
Fim2- 1	100	100	100
Fim3- 1	100	100	70
2	0	0	29
3	0	0	1
****Serotype 1**	20	0	0
1,2	40	50	37
1,3	20	17	63
1,2,3	20	33	0

*Prn allele type was determined for just 76 ACV era strains due to poor mapping of reads in this region in 8 strains.

** Serotype was not determined for one ACV era strain, thus frequencies are based on 83, not 84, strains in this era.

4.4 SNPs Specific to ptxP3 Strains

PtxP3 strains are the predominant allelic type in current circulation and appear to have different infection biologies compared to *ptxP1* strains. The *ptxP3* SNP itself appears to be both a direct cause of these differences and a marker for other genetic variations that contribute to them (King *et al*., 2013). To investigate the genetic traits of UK *ptxP3* strains, SNPs specific to this lineage were identified. In total, 22 such SNPs were identified (Table 4.2). Ten were intergenic, seven of which were in the direct repeat region of IS elements which are present in multiple copies in the *B. pertussis* genome. It is not clear if these particular IS elements are functional. Twelve SNPs were in coding regions, of which, seven were non-synonomous mutations (NSM) and 5 were synonymous (SM). The 7 NSM were in genes within the "transport and binding proteins", "pseudogene", "conserved hypothetical", "virulence-associated", "regulation" and "unknown" functional categories as defined previously (Parkhill *et al*., 2003). All of these SNPs were also identified among the global panel of *B. pertussis* strains (Bart *et al*., 2014). However, of the 22 SNPs identified here as being *ptxP3*-specific, only 10 were identified as being *ptxP3*-type-specific in the previous study (Table 4.2), the other 12 SNPs were also identified among non-*ptxP3* strains globally.

Table 4.2. SNPs specific to UK *ptxP3* strains.

Location[a]	Type[b]	Mutation[c]	Global *ptxP3*[d]	Details
36857	INT	A:G	Yes	93 bp upstream of BP0032 (encoding a putative transport protein), 156bp upstream of BP0033 (encoding GlyQ-glycyl-tRNA synthetase alpha chain)
617083	INT	T:G	No	within the 5' repeat region of IS481 (BP0611). 31bp upstream of transposase start codon.
617084	INT	C:T	No	within the 5' repeat region of IS481 (BP0611). 32bp upstream of transposase start codon.
1077844	INT	C:T	No	within the 5' repeat region of IS1663 (BP1035). 139bp upstream of transposase start codon.
1170424	INT	A:G	No	within the 5' repeat region of IS481 (BP1114). 31bp upstream of transposase start codon.
1222400	INT	A:C	No	within the 5' repeat region of IS481 (BP1157). 31 bp upstream of transposase start codon.
1635654	INT	T:G	No	within the 5' repeat region of IS481 (BP1557). 31 bp upstream of transposase start codon.
2259917	INT	G:C	No	within the 5' repeat region of IS481 (BP2135). 98 bp upstream of transposase start codon.
3263622	INT	A:C	Yes	193 bp away from BP3062. Putative integral

				membrane transport protein.
3988168	INT	G:A	Yes	89 nucleotides away from the start codon of ptxA. ptxP3allele.
196307	NSM	T:C	Yes	BP0194. Putative transport protein.
299559	NSM	C:T	Yes	BP0292. Pseudogene. Conserved hypothetical protein.
1331840	NSM	G:A	Yes	Pseudogene. BP1261. Hypothetical protein.
1547488	NSM	A:G	No	BP1471. Conserved hypothetical protein.
2374322	NSM	T:C	Yes	BP2249. BscI. Type III secretion apparatus protein.
2651008	NSM	G:A	Yes	BP2502. Hypothetical protein.
3134458	NSM	G:C	No	BP2946. Probable transcriptional regulator.
185405	SM	G:A	No	BP0184. Putative periplasmic protein.
518837	SM	T:C	No	BP0507. Putative membrane protein.
694521	SM	A:G	Yes	BP0678. Putative peptide chain release factor.
3840411	SM	G:A	Yes	BP3630. RpsH. 30S ribosomal protein.
3991376	SM	C:T	No	BP3787. PtxC. Pertussis toxin subunit protein.

[a] Tohama I reference genome coordinates (accession no. BX470248) .

[b] Int: SNP is in an intergenic region. NSM: non-synonymous mutation, SM: synonymous mutation.

[c] e.g. C:T – C to T mutation.

[d] SNP is also defined as *ptxP3*-specific in study of global *B. pertussis* population (Bart *et al*., 2014).

4.5 SNP Rates are high in Vaccine Antigen Encoding Genes

Previously, it was identified that genes in the 'cell surface' functional category had higher SNP densities than the *B. pertussis* chromosomal average (Bart *et al*., 2014). However, ACV vaccine-mediated immunity is exerting selective pressure primarily on the proteins used in these vaccines and might be driving their evolution. To explore this, the SNP density (SNPs per bp) for the 9 ACV antigen genes (Ptx comprises five different proteins) and for the other 591 genes comprising the 'cell surface' category was calculated for all strains within each vaccine era and compared. Secondly, it was investigated if the SNP rate in ACV genes had increased since the introduction of ACVs. The 'cell surface' category is considered the most compatible group of proteins for comparison work. They are exposed to the bacterium's environment, including the host during infection, and thus will experience the same environmentally-derived selection pressures as the ACV antigen encoding genes except that they will not be subjected to ACV-induced immunity. Thus, differences in evolution between these two categories can be ascribed to ACV-induced selection pressure.

The difference in mean SNP density across genes within the two samples (mean SNP density in vaccine antigen genes minus mean SNP density in cell surface genes) was calculated. A non-parametric Monte Carlo simulation was used to assess the significance of this difference by determining how often a difference as large or larger than this was derived by randomly resampling two samples the same size as above, from the pool of vaccine antigen and cell surface genes. This revealed that in each era, vaccine antigen encoding genes had significantly higher SNP densities than other cell surface genes ($P<0.05$, Table 4.3), with the difference being greatest among ACV-era strains. This suggests that the vaccine antigen genes are faster evolving than other surface protein encoding genes, and that they were also faster evolving even prior to the introduction of widespread vaccination.

To compare SNP densities in vaccine antigen genes between eras, SNP densities within each era were normalised by dividing by the mean SNP rate across all of the genes concerned (ACV antigens and cell surface). In comparison to the prior analysis this has less power owing to the much smaller sample of ACV genes compared with total cell surface genes. Although the normalised SNP density in ACV-era strains was greater than in pre-ACV era strains, the difference was not statistically significant, $P=0.160$. However, the number of pre-ACV strains in this analysis was small. Thus, the same analyses were repeated using SNP data from the global collection of strains, for which the year of

isolation was known (Bart *et al*., 2014), and incorporating the UK strains sequenced here (see Table 4.3). Again, a significantly greater SNP frequency was found in ACV antigen genes than other cell surface genes, in all three eras. This time, there was also a significantly higher SNP frequency in ACV genes among ACV era strains compared to pre-ACV era strains (P=0.0177) suggesting that the relative SNP density in ACV antigen genes has increased since the introduction of ACVs. These results suggest that ACV genes are intrinsically fast evolving and provide some support for the hypothesis that they are even faster evolving since the introduction of ACVs.

The more rapid evolution in the ACV antigen genes could be due to either a higher underlying mutation rate or different selection at the protein level. The different selection could be positive selection or weaker purifying selection. To distinguish between these two possibilities, SNPs were split into SM and NSM. High NSM but not SM rates would suggest altered protein-level selection. A higher rate of synonymous evolution (with possibly a weak non-synonymous effect) would suggest higher mutation rates. Interpretation here is difficult owing to well-described but incompletely understood correlation between synonymous and non-synonymous rates.

Among WCV- and ACV-era global strains, but not pre-vaccine era strains, the SM frequency was significantly higher in ACV antigen genes compared to other cell surface genes, Table 4. When comparing ACV-era to pre-ACV era strains, the SM frequency in ACV antigen genes was significantly higher (P=0.004). NSMs also occurred at significantly greater frequency in ACV antigen genes compared to other cell surface genes (Table 4.4). The magnitude of this effect is greater than that seen for SMs suggesting the higher evolutionary rate of ACV antigen genes compared to cell surface proteins is largely owing to protein-level selection on the antigens. Evidence for a strong recent increase is less clear-cut. When comparing strains from the ACV-era to pre-ACV era strains, the NSM frequency in ACV antigen genes was on the edge of significance (P=0.051). Overall, these results provide support for the hypothesis that the genes encoding antigens chosen for ACVs are intrinsically fast evolving, in part owing to selection on their antigenic products. However, the possibility that in the ACV-era there has been an increase in the mutation rate cannot be formally discounted (see discussion below).

Table 4.3. SNP rates in vaccine antigen encoding genes compared to other cell surface genes for the different vaccine eras among UK strains and globally.

Vaccine era. (No. of strains)	mean SNP/bp vaccine antigen genes	mean SNP/bp cell surface genes	Diffference (vaccine antigens - cell surface)	Difference normalized (Difference/mean SNP density)	P (SNP rate vaccine antigens > SNP rate cell surface)
UK Pre-1920-1956 (5)	3×10^{-4}	7.8×10^{-5}	2.22×10^{-4}	2.72	0.045
UK WCV 1957-2000 (6)	4.75×10^{-4}	5.9×10^{-5}	4.17×10^{-4}	6.40	0.016
UK ACV 2001-2012 (84)	1.73×10^{-3}	1.55×10^{-4}	1.57×10^{-3}	8.82	0.0004
Global Pre-1920-1956 (19)	1.45×10^{-3}	5.85×10^{-4}	8.62×10^{-4}	1.44	0.012
Global WCV 1957-2000 (204)	2.62×10^{-3}	1.01×10^{-3}	1.61×10^{-3}	1.56	0.002
Global ACV 2001-2012 (188)	2.91×10^{-3}	4.23×10^{-4}	2.49×10^{-3}	5.41	0.0001

Table 4.4. Synonomous (SM) and non-synonomous (NSM) mutation rates in vaccine antigen genes compared to other cell surface genes among strains isolated during the different vaccine eras.

Vaccine era. (No. of strains)	mean SNP/bp vaccine antigen genes	mean SNP/bp cell surface genes	Diffference (vaccine antigens - cell surface)	Difference normalized (Difference/mean SNP density)	P (SNP rate vaccine antigens >SNP rate cell surface)
SM Global Pre-1920-1956 (19)	1.32×10^{-4}	2.4×10^{-4}	-1.07×10^{-4}	-0.45	0.627
SM Global WCV 1957-2000 (204)	9.66×10^{-4}	4.23×10^{-4}	5.43×10^{-4}	1.26	0.045
SM Global ACV 2001-2012 (188)	9.68×10^{-4}	1.76×10^{-4}	7.92×10^{-4}	4.20	0.011
NSM Global Pre-1920-1956 (19)	1.18×10^{-3}	3.40×10^{-4}	8.38×10^{-4}	2.38	0.006
NSM Global WCV 1957-2000 (204)	1.96×10^{-3}	5.83×10^{-4}	1.37×10^{-3}	2.28	0.002
NSM Global ACV 2001-2012 (188)	1.95×10^{-3}	2.38×10^{-4}	1.71×10^{-3}	6.48	0.0002

4.6 Regions of Difference

Deletions have been a major feature of *B. pertussis* evolution and appear to be on-going (Parkhill *et al*., 2003; Caro *et al*., 2008). Compared to the Tohama I reference genome, most of the major deletions observed among the strains analysed here had been identified previously (Caro *et al*., 2008). Numerous small deletions were found in only a few, or just one isolate, suggesting that deletion of DNA is common among *B. pertussis* strains. Interestingly, some deletions appeared specific to the UK *ptxP3* strains, but no deletions specific to outbreak isolates were detected (Appendix B).

Regions from individual strains that were not present in the Tohama I reference genome were investigated by BLAST analyses. These regions were also found within other *B. pertussis* genomes (BP18323 and CS), or in *B. bronchiseptica* RB50, similar to that reported in other studies (Kallonen *et al*., 2011). Thus there were no novel insertions or gene acquisition among the outbreak isolates.

4.7 Discussion

The resurgence of pertussis in countries with high levels of vaccination has caused widespread concern. Among other factors, *B. pertussis* evolution away from efficient control by vaccine-induced immunity has been proposed as a contributor to this. Recently, whole genome sequencing was used to define global genetic variability among *B. pertussis* isolates and this identified genetic changes in the *B. pertussis* population over time (Bart *et al*., 2014).

The genomes of UK *B. pertussis* isolates have been analysed extensively, with emphasis on strains from the 2012 outbreak. For the first time it has been shown that many genetically distinct *B. pertussis* strains contributed to this outbreak and importantly, that it was not due to the emergence of a novel, hypervirulent clone or expansion of an individual lineage. Furthermore, outbreak strains were genetically very similar to those circulating during periods when the incidence of pertussis was low. There appeared to be no significant association between groups of strains holding particular SNP types and host age or geographical origin. The vaccine status was not known for all hosts, and thus it was difficult to investigate an association between vaccine status and these grouped strains. However those strains isolated from the unvaccinated one-month-old group, did not cluster on the phylogenetic tree, which suggests this lack of association.

The *ptxP3* type is dominant world-wide and UK outbreak strains are also predominantly of this type. Analysis of global isolates identified just 19 SNPs as *ptxP3*–specific (Bart *et al*.,

2014). Here, 22 SNPs distinguished *ptxP3* from *ptxP1* strains. However, just 10 of these were common to both sets of *ptxP3*-specific SNPs. If *ptxP3* strains have increased fitness or virulence compared to older isolates, the analysis here suggests that very few SNPs are responsible for this, or that particular combinations of SNPs are important, only some of which are *ptxP3*-specific. Overall, these data argue against large-scale genetic changes being behind the recent resurgence in pertussis.

Changes in alleles of the genes encoding vaccine antigens have been well documented (for example, van Gent *et al*., 2012) and supports the hypothesis that selection pressure from ACV induced immunity is a driver of *B. pertussis* evolution. However, definitive studies to demonstrate that allelic variation enhances evasion of vaccine-mediated immunity are lacking and particularly difficult to perform given the inability to conduct studies with human hosts and that studies using animal models struggle to detect subtle changes and will not include population level effects that are certainly important for selection of variants among *B. pertussis* worldwide. Here, compelling evidence is provided showing that genes encoding ACV antigens are evolving more rapidly than other cell surface genes (which are considered the most suitable comparator group), containing a significantly higher frequency of SNPs in each of the vaccine eras. Interestingly, this was true even in the pre-vaccine era. It is likely that even in the absence of vaccination, the natural immune response to these antigens creates selective pressure, particularly for a pathogen that is restricted to the human respiratory tract. Studies have shown this to be true of the *Plasmodium falciparum* whereby epitopes in proteins that interact with the immune system were under stronger diversifying selection (Krzyczmonik *et al*., 2012).

Of particular importance is the calculation that ACV antigen gene evolution rates have increased significantly since the introduction of ACVs. This study is the first demonstration of this effect. This suggests that the use of ACVs has increased selection pressure on ACV antigens, selecting for ACV antigen gene variants. However, while the frequency of SM in ACV antigen genes was significantly higher in ACV era strains compared to older strains, the frequency of NSM was on the edge of significance (P=0.051). In turn this suggests that selection pressure from vaccine-mediated immunity is not the sole driving force for ACV antigen gene variation. A different interpretation is that the mutation rate of ACV antigen genes has increased since the introduction of ACVs. If synonymous sites are under weak purifying selection (i.e., not perfectly neutral), then there is a lag between a SNP arising and its elimination by this selection, resulting in an excess of SNPs in the modern era. However, normalising ACV gene SNP rates by the SNP rates

for all genes within the era largely eliminates this effect (i.e., SMs in cell surface genes should be equally over-represented in the modern era). However, if SMs in ACV genes and cell surface genes are under different intensities of purifying selection, then this result could be found.

Either way, the more rapid evolution at the protein level (as determined by NSM) of ACV proteins compared to other cell surface proteins, across all eras suggests that strains will become increasingly mismatched to those used for vaccine production and this could lead to decreased vaccine efficacy over time.

There are limitations arising from the sample sizes used here. Very few older strains, particularly from the pre-vaccine era, were available and this limited the power of the analyses here, even though the difference in SNP rates between ACV antigen genes and cell surface protein genes was statistically significant. To counteract the limited strain collection available for UK strains, the analyses were expanded to include a global collection of strains. This expanded set of strains increased the power of the analyses and resulted in a greater significance of the results. Monte Carlo simulation was used here as it produces an unbiased estimation of probability. This approach requires no approximations as it uses only observed values and does not require assumptions of normality or even any assumptions about the distribution of data. Thus Monte Carlo simulation is considered to be the gold standard for unbiased estimations of 'P', and superior to other possible approaches. One issue with this approach is that it is likely to produce slightly different answers each time it is used on a dataset, due to the random sampling of the data involved. However, it is generally considered that performing sufficient randomisations eliminates this problem and 10 000 iterations is considered sufficient for this.

Strains were assigned to one of three eras: pre-vaccine, WCV or ACV in an attempt to associate changes in evolution rates with vaccination and the type of vaccine used. There is not a clear cut-off between WCV- and ACV-eras as in most countries that use ACVs, there were several years in which the primary course of vaccinations were with WCV while ACVs were used as boosters. In the UK, both were in use from 2001 until 2004. However, just one strain, UK15, isolated in this period was included in this study and inclusion in either ACV or WCV eras does not affect the result of the analysis.

It is perhaps surprising that such a statistically significant increase was observed in the rate of evolution of ACV antigen encoding genes in the ACV-eras compared to the WCV-era as it is a short time since the introduction of ACVs. However, the very recent sudden rise in the frequency of Prn-deficient strains has been taken as evidence of a strong ACV-

induced selection pressure. Further analysis could subdivide strains in this ACV-era to compare the rates of evolution over time upon the introduction of these vaccines, with future analyses incorporating the most recent isolates. Care must be taken to consider the type of ACV used in different countries if using global data as some vaccines contain Prn, FHA and PT, whereas others also contain Fim2 and Fim3 (and in Denmark the vaccine contains just PT). It is clear that the ACV antigens are not a homogenous group as PT and FHA are secreted antigens, whilst Prn and Fim are integral to the outer membrane. However, they were selected for inclusion in ACVs in the 1980s based on their immunogenicity in the WCV and demonstration of protection in mouse disease models (Robinson *et al*., 1985). The ACV antigens were chosen on the basis of their immunogenicity and it is likely that this property has driven the relatively high evolution rates of the genes encoding these antigens. These results raise fresh concerns over the ability of current acellular pertussis vaccines to continue to control disease.

There is no doubt that the vaccine has provided a high level of protection and prevention of pertussis disease in the human population, however it has not succeeded in breaking the chain of infection by preventing transmission. As a result, *B. pertussis* strains still circulate throughout the population, which is in contrast to other vaccine-preventable diseases such as diphtheria. Thus, it could be argued that evolution of *B. pertussis* strains away from vaccine-induced immunity is inevitable due to exposure to this selective pressure during circulation. Globally, vaccination against pertussis varies, including the type of vaccine used and the schedule of vaccinations. Furthermore, different coverage levels are reached. This could lead to variation in the level of exposure of strains to vaccine-mediated immunity and thus variation in the rate of evolution in strains worldwide. As well as suggesting that *B. pertussis* evolution has accelerated since the introduction of vaccination, Xu *et al* (2015) found that the "molecular clock" of *B. pertussis* isolates varied between countries concluding that these differences might have been due to the variation in vaccine coverage and schedule between countries (Xu *et al.*, 2015).

The results here indicate that the rate of evolution has increased upon the replacement of the WCV with the ACV, suggesting that the selective pressure on these antigens has intensified owing to the specificity of the ACV against these proteins. However there is no evidence that genetic changes caused the outbreak. Phenotypic variation in response to selective pressure can take a number of years to accumulate and establish itself in a bacterial population before the effects are seen**,** as observed for the rise and spread of antimicrobial resistance. Eleven years on since the introduction of the ACV in the UK

and six years in the infant schedule, a resurgence of pertussis was seen. It could be that this is the number of years it has taken for these structural alterations to accumulate in the *B. pertussis* population before the affects are seen.

Data suggest that vaccine-induced anti-Prn and anti-FHA antibodies provide the most opsonophagocytosis activity (Aase *et al.*, 2011). If strains are evolving to be recognised at a lower level by such antibodies, immune mechanisms such as opsonophagocytosis may be less effective. Identifying differences in recognition between outbreak and pre-outbreak strains could highlight a possible fitness advantage in strains that were part of the resurgence in the UK.

The increase in Prn-deficient strains is perhaps the best evidence for evolution and adaptation of *B. pertussis* strains away from vaccine-induced immunity, however only one Prn-deficient strain was identified in this UK collection. To date no research has investigated different levels of Prn expression between strains, as a reduction in Prn expression may also provide an evasion strategy. There are a number of mechanisms that could give rise to gene expression variation, SNPs being one of them. However no mutations specific to the outbreak strains were seen in *prn.* Therefore, the next step of the thesis was to explore the expression levels of the Prn antigen and recognition level of strains by IgG binding of post-vaccine sera.

The data presented in this chapter has been published in the Journal of Infectious Diseases and is included in Appendix C.

5

Results

Pertactin expression and IgG binding to *B. pertussis* using post acellular vaccine sera

5.1 Rationale

Pertactin is considered an important antigen of ACVs. The large increase in Prn-deficient strains worldwide has led to the theory that this absence provides a fitness advantage to *B. pertussis* strains. The genetic data from the UK *B. pertussis* clinical strains identified that only one was Prn-deficient, seemingly a much lower incidence than other countries experiencing resurgence. The recent increase in frequency of Prn-deficient isolates, and the demonstration that Prn-deficient strains have increased fitness compared to those expressing Prn in immunized hosts (albeit in a mouse model) fits with the hypothesis that avoidance of immunity directed against Prn is a selection pressure bearing on *B. pertussis*. An additional mechanism that might operate to avoid this immunity is a reduced level of expression of Prn, as opposed to Prn-deficiency. However, this has not yet been investigated.

This chapter aims to test the hypothesis that in addition to Prn deficiency there are variable levels of Prn expression among strains, which results in variable levels of recognition of strains by antibodies in immune sera.

5.2 Approach

A panel of 2012 outbreak strains and historical (pre-outbreak) strains were selected encompassing a range of different SNP profiles and thus genetic variation as identified in the previous chapter. The level of Prn expression by these strains and recognition by post-vaccine sera was then measured. Prn expression in *B. pertussis* strains was determined by a surface localised antibody assay. Anti-Prn monoclonal antibody (Mab) was added to wells containing *B. pertussis.* A secondary antibody conjugated with fluorescein isothiocyanate (FITC) was then added to form a complex with the anti-Prn Mab bound to Prn on the bacterial surface. The resulting fluorescence of individual bacteria was measured by flow cytometry. Determining IgG binding to strains with post-vaccine sera

was performed using a similar assay. Individuals were vaccinated with the Repevax® booster vaccine, a 5-component vaccine containing the antigens Prn7, PtxA3, Fim 2/3 and FHA from the *B. pertussis* strain BP10536. Serum was added to the panel of *B. pertussis* strains, followed by the addition of anti-human Mab FITC conjugate. The level of fluorescence minus the conjugate control (FI-C – see Materials and Methods), and therefore bound antibody conjugate, was quantified using a flow cytometer. This FI-C reading indicated the amount of Prn on the surface of strains and bound post-vaccine IgG to the surface of the *B. pertussis* strains.

5.3 Pertactin expression in clinical strains

Pertactin expression was characterised by the binding of an anti-Prn Mab to formaldehyde fixed *B. pertussis* strains. All strains were prepared and analysed using the same protocol so that differences resulting from culture or sample preparation conditions were minimized. The well characterised laboratory-adapted strains Wellcome 28 and Tohama I together with Arg37, a Bvg$^-$ phase-locked variant of Wellcome 28 that expresses very low levels of vaccine antigens, was used as internal control strains. Anti-Prn Mab binding was repeated on three separate occasions producing three datasets. The three datasets correlated well producing an R-value of 0.92, 0.89 and 0.86 when comparing dataset 1 with 3, dataset 1 with 2, and dataset 2 with 3, respectively. As fluorescence values obtained varies from day to day data were normalized using values obtained using three strains common to these three experiments. The mean ratio of fluorescence values from these strains compared with experiment 1 was used to normalize data from experiments 2 and 3 to experiment 1 and the results are shown in Fig 5.1. The coefficient of variation (Reed *et al*., 2002) (%CV) of Prn expression for strains that were repeated on separate occasions was calculated (Table 5.1) and these values are less than 35% which is acceptable for an antibody binding assay and was thus considered reproducible. The UK8 clinical strain isolated from 1979 showed the highest mean level of anti-Prn Mab binding, closely followed by UK3 (1946), UK39 (2012) and UK48 (2012). A Prn deficient strain, UK50, was identified in the genetic data, which was confirmed in this assay. This strain had similar levels of Prn expression to Arg37. Due to the possibility that UK50 may have been Prn negative, it was not included in the statistical analyses of determining differences in Prn expression between strains grouped in to 'outbreak 2012' and 'historical' strains.

Table 5.1 The coefficient of variation (Reed *et al*., 2002) (%CV) of FI-C readings, which are directly proportional to Prn expression, in strains, which were analysed on separate occasions. Note a %CV value was not calculated for some strains as they were contained in one dataset only.

Clinical strain	%CV
UK88	17.3
UK87	1.6
UK8	10.8
UK56	0.2
UK48	9.6
UK31	27.6
UK3	16.6
UK29	3.1
UK27	29.0
UK23	9.0
UK17	3.7

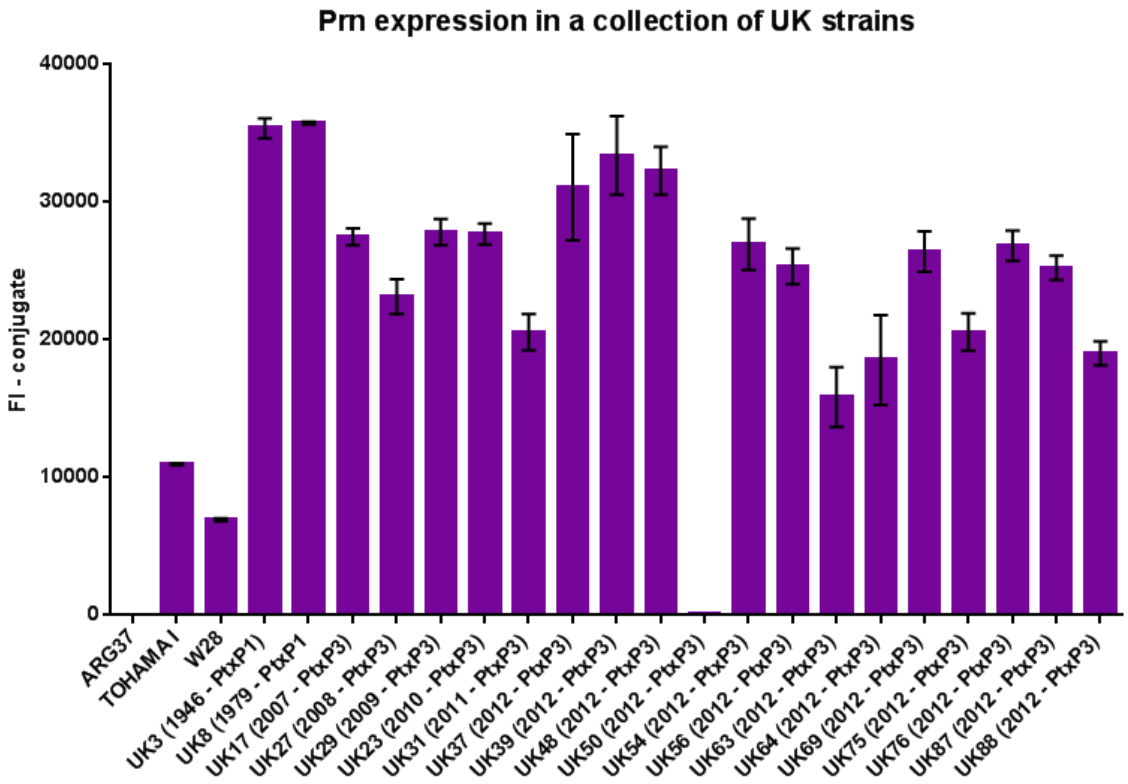

Fig.5.3.1 The mean values of fluorescence of duplicate wells and the standard error bars of anti-Prn Mab binding to a collection of *B. pertussis* strains.

Differences in Prn expression between historical (strains previous to the 2012 outbreak) and outbreak strains (isolated from 2012) as well as between *ptxP1* and *ptxP3* strains were analysed using an unpaired, two-tailed, t-test and the data plotted in a Fig.5.3.2a & b respectively. No significant difference was seen between historical and 2012 outbreak strains (p-value =0.26). However, there was a significant difference in the level of Prn antigen expression between *ptxP1* and *ptxP3* strains (p-value =0.01). However, only two strains were in the *ptxP1* group, compared to 17 strains in the *ptxP3* group. Thus this data is preliminary and further analysis with more strains representing *ptxP1* is required before definite conclusions can be drawn.

Fig.5.3.2a

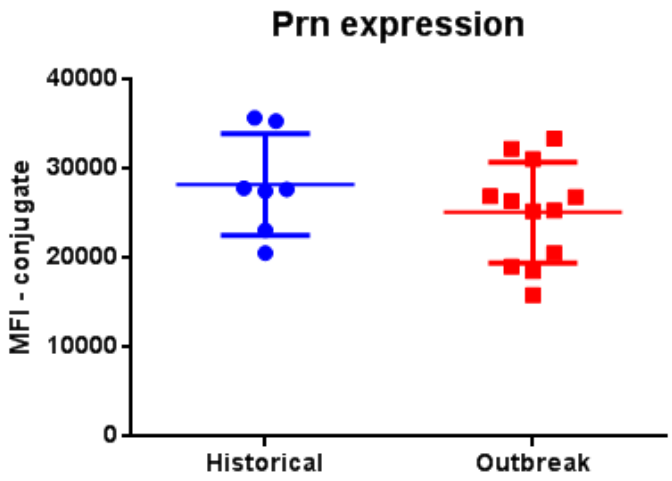

Fig 5.3.2b

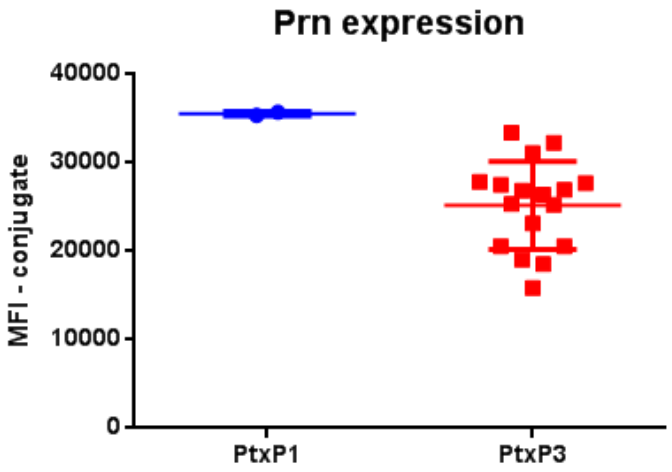

Fig 5.3.2a&b Box plot identifying the mean level of anti-Prn Mab binding to the surface of historical (1946-2011) and outbreak (2012) strains (**a**) and *ptxP1* and *ptxP3* strains (**b**).

5.4 Differences in recognition of outbreak and historical strains by post-vaccine sera

Post-vaccine sera were used to investigate recognition of outbreak and historical strains by vaccine-induced antibodies (Fig. 5.4.1). The experiment was repeated to test for reproducibility. The correlation between the two sets of data was strong (r = 0.75, p-value = >0.001; Fig. 5.4.1).

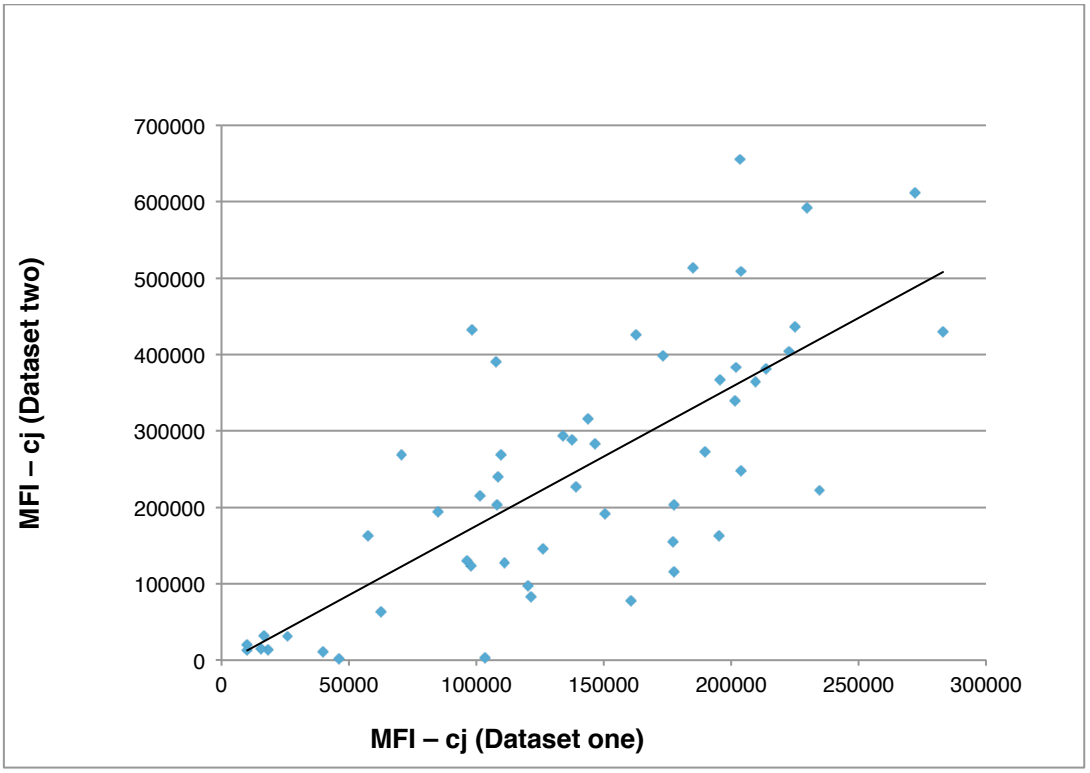

Fig.5.4.1 Correlation (r = 0.75) between the two sets of data used to detect the recognition of clinical strains by post-vaccine sera. A line of best fit is shown.

Arg37, the Bvg-phase negative control, bound the lowest level of antibody (Fig.5.4.2) with a geometric mean of 36,818 FI-C. Tohama I, the laboratory-adapted strain also bound low levels of antibodies. The Prn-deficient strain UK50 had the lowest level of anti-Prn Mab binding compared to the other clinical strains, at an FI-C of 61,529. Those strains that bound the highest level of IgG were isolated in 2012 (UK48), as well as in 1946 (UK3) (Fig5.4.2). The level of anti-Prn Mab binding appeared not to correlate with year of isolation (p-value =0.79) (Fig.5.4.3b). Likewise, there were no differences in IgG binding with post-ACV sera between *ptxP1* and *ptxP3* strains (p-value =0.35) (Fig.5.4.3a). UK50 was not used in this analysis due to its likely Prn- deficiency. However, as stated above, the use of only 2 *ptxP1* strains in this analysis makes any interpretation preliminary and further analysis with a larger strain panel is required.

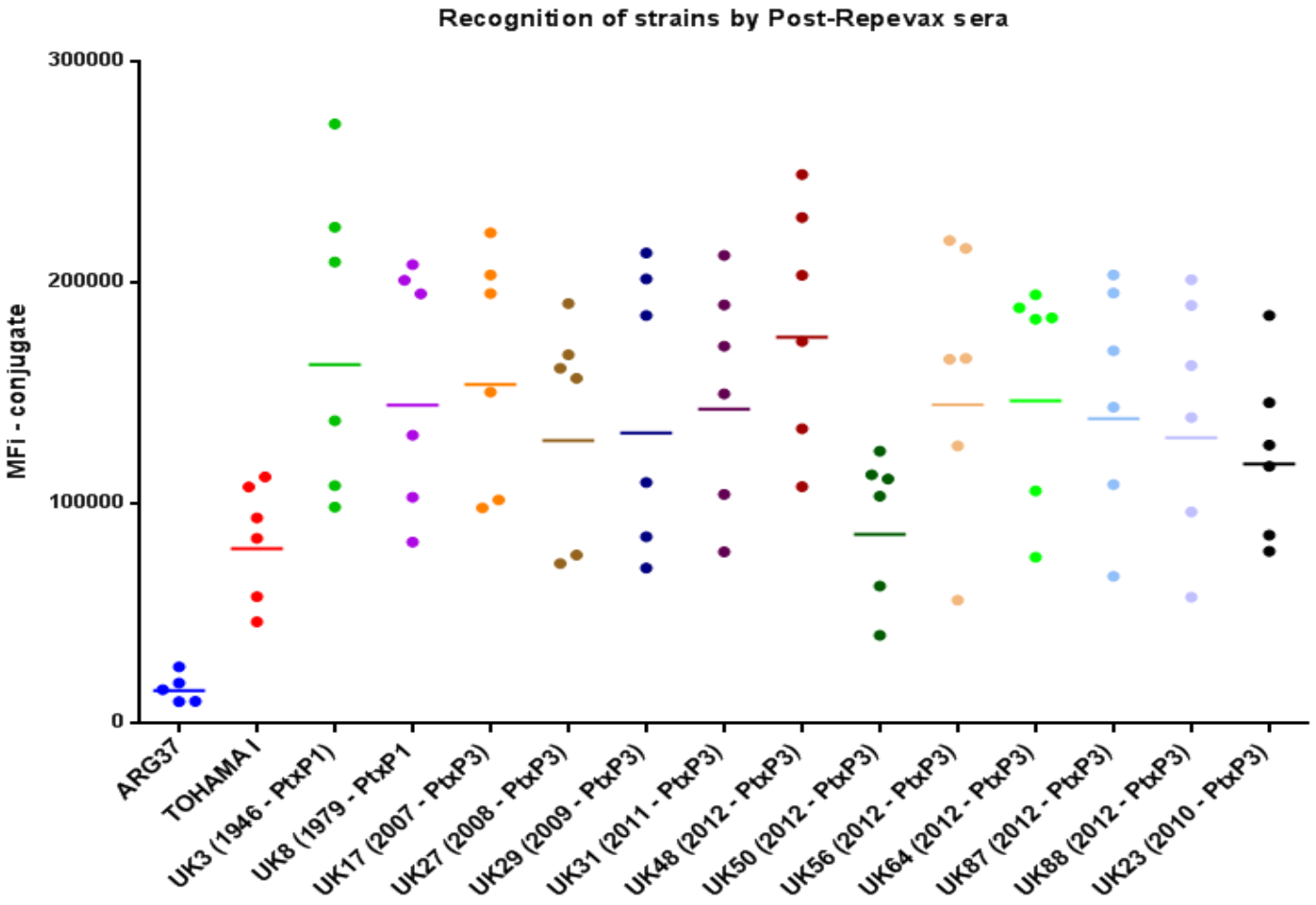

Fig.5.4.2 The mean values of fluorescence obtained with duplicates of the 7 post-vaccine sera, comparing the quantity of IgG bound to the surface of each strain. The thick bold lines on each plot represent the geometric mean of all seven sera combined. Arg37 is a negative control and Tohama I is a positive control.

Fig.5.4.3a

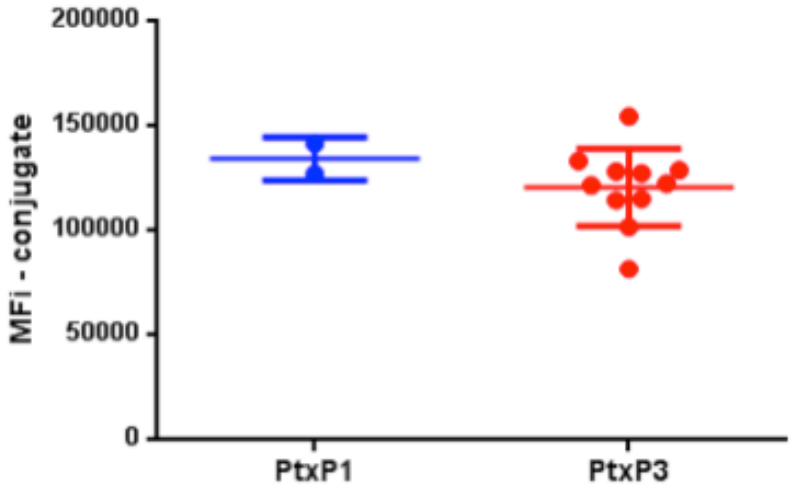

Fig.5.4.3.b

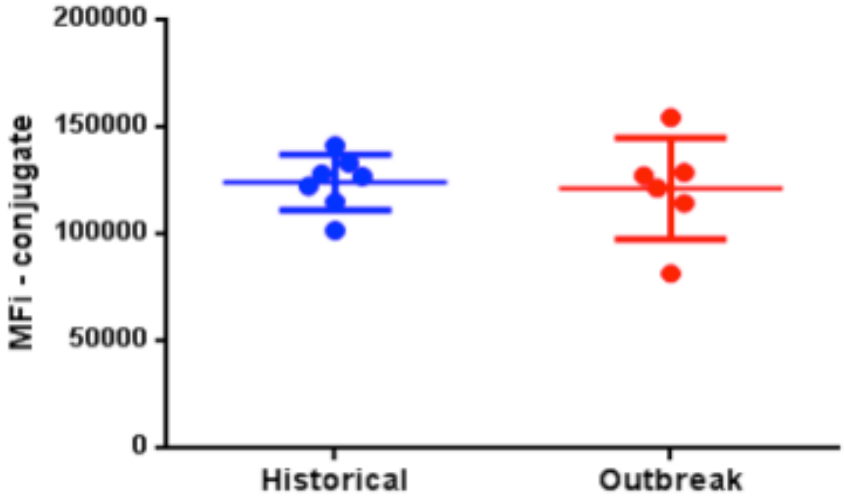

Fig 5.4.3a&b IgG in post ACV serum bound to and *ptxP1* and *ptxP3* strains (**a**) and historical (1946-2011) and outbreak (2012) strains (**b**)

5.5 Relationship between Prn expression and IgG bound to *B. pertussis* with post-ACV sera

The correlation between Prn expression and IgG bound to *B. pertussis* using post ACV sera was investigated. Identifying a positive correlation between the two variables could suggest that anti-Prn antibody contributes to a large proportion of overall binding and variability in Prn expression could lead to variation in IgG binding with post ACV sera. Data from some strains that were used in the Prn expression studies have been omitted from these analyses because of failed readings on the flow cytometer when the post-vaccine sera assays were performed. Therefore, only those strains where the coefficient of variation (CV) between duplicates was $\leq$ 35% were included. A positive correlation between prn expression and recognition by post-vaccine sera (r =0.75, p-value = >0.001) was observed, and the line of best fit is shown in Fig.5.5.1. This analysis includes the Prn- strain UK50. If this is removed the correlation reduces to the weaker value r =0.46 (p-value = 0.1). The relevance of this finding and the case for including Prn- strain in the analysis is discussed below.

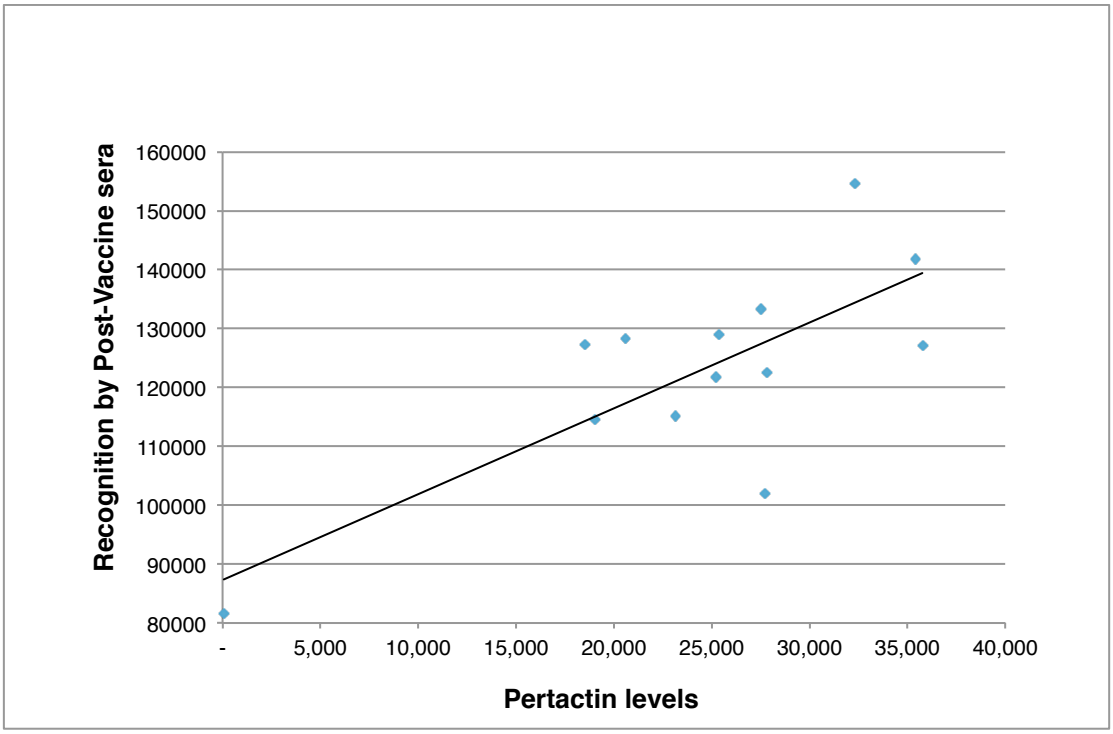

Fig.5.5.1 Correlation between the level of anti-Prn Mab binding and IgG binding with ACV-induced antibodies. A line of best fit is shown.

5.6 Discussion

The genetic analysis of the 95 UK strains established that many genetically distinct, but closely related strains contributed to the 2012 UK epidemic. This argues against large-scale genetic changes being behind the resurgence (see Chapter 4, page 36). Pertactin has received much attention due to the increasing numbers of Prn-deficient strains circulating worldwide. Selective pressure from vaccine-mediated immunity has been suggested to be a driver for the emergence of Prn-deficient strains. Prn is classed as one of the key components of the vaccine as the level of antibodies against this antigen has shown correlation with protection in household contact studies (Storsaeter *et al*., 1999; Cherry *et al*., 1998; Olin *et al*., 2001; Higg *et al*, 2012), and thus the selective pressure on this particular protein may be strong, possibly stronger than other antigens contained in the vaccine, giving a possible explanation for the increasing number of Prn-deficient strains.

The genetic analysis revealed one strain, UK50, to be Prn-deficient. This was confirmed in this Prn expression study. It is not clear why only one Prn-deficient strain was identified among UK strains, as other countries have observed a far greater incidence. It is possible that the sample size here was not a large enough to identify more Prn negative strains. However, the proportion of Prn-negative strains in other countries has been reported as high as >50%. Here, sixty-one 2012 strains were analysed, with just one Prn-deficient strain identified (1.6 %), suggesting that the incidence of Prn-deficiency is low.
In previous studies, No attention has been given to the level of Prn expression in Prn-expressing strains, thus the main focus here was to analyse potential differences in the expression level of Prn in outbreak and historical strains.

Overall, the data did not indicate that Prn expression differed between historical and outbreak strains. Similarly, post ACV serum antibodies bound to outbreak and historical strains at a similar level. Therefore, it can be concluded that there is no clear phenotypic difference in Prn expression or binding of post ACV antibodies to the 2012 outbreak strains and historic strains. However, the significant difference in Prn expression between *ptxP1* and *ptxP3* strains is a very interesting observation. The anti-Prn Mab binding to *ptxP3* strains was lower, albeit that only 2 *ptxP1* strains were analysed. This lower Prn expression could be a fitness advantage, in agreement with King *et al* (2013) who found that the genetic background of *ptxP3* strains improved overall fitness and colonization in mice. In that study they showed that *ptxP3* strains expressed a number of virulence genes at a higher level than the *ptxP1* strains, but interestingly, *prn* was not one of them suggesting complex control of the expression of individual genes.

The positive correlation observed between Prn expression and post ACV IgG binding supports the hypothesis that lower Prn expression leads to reduced IgG binding with post ACV sera. This analysis included UK50, which lacks Prn expression. Although the concentration of anti-Prn IgG in the sera used in this study could not be obtained, the low level of post ACV IgG binding observed with this strain shows that anti-Prn IgG is an important component of opsonizing antibodies following ACV vaccination. This is perhaps unsurprising as Prn is the only integral membrane protein among the ACV antigens, with Ptx and FHA mostly excreted proteins. Also, the strains analysed predominantly express Fim3 only and the 5-component ACV has been shown to elicit a poor IgG response to Fim3 (Hallander *et al*., 2014). This suggests that evolution of strains towards avoidance of vaccine-induced immunity involves avoidance of antibody binding to Prn. The appearance of Prn-deficient strains at high frequency is a recent phenomenon and fits with the switch to ACVs from WCVs increasing the immune selection pressure on Prn-expression among strains. The focus of this immune pressure is demonstrated by the importance of anti-Prn IgG for binding to *B. pertussis* shown here. In this study there is only weak evidence that decreased expression of Prn is evolving among recent strains perhaps suggesting that complete abrogation of Prn expression is required to produce a selectable fitness advantage in a population with high ACV coverage.

Investigating the correlation between expression levels of the remaining four vaccine antigens Ptx, FHA, Fim2 and Fim3 and recognition by post-vaccine sera would enable comparative work in order to fully identify the extent to which anti-Prn antibodies determine overall recognition of bacteria by vaccine-induced antibodies. Before any comparisons between these five antigens could be made however, identifying the antibody titers of anti -Ptx, -FHA, -Prn, -Fim2 and -Fim3 IgG in patient sera would be necessary. However due to time and money constraints, this was not possible.

In summary, the phenotypic data thus far have not identified a level of Prn expression or vaccine IgG binding that is specific to the 2012 outbreak strains. The finding that binding by post-ACV IgG and expression of Prn antigen have a positive relationship is a very interesting observation and provides further evidence that vaccine-induced antibodies are playing a role in the evolution and evasion of *B. pertussis* strains in vaccinated populations.

6

Final Discussion

The data from this project argue against large-scale genetic changes as a contributor to the UK 2012 pertussis resurgence. Ongoing genetic drift in *B. pertussis* strains is a major part of its evolution (Xu *et al*., 2015). Data have shown that there are generally a number of distinctive genetic profiles present at any one time and such profiles are on-going (Cassiday *et al*., 2016). In light of this, the lack of a distinctive clone in the 2012 outbreak panel is not surprising. However, this project identified that the genes coding for the vaccine antigens are evolving at an accelerated rate, which was further increased upon the introduction of the ACV. This supports the hypothesis that strains are evolving to evade vaccine-induced immunity and similar studies have identified the evolution of *B. pertussis* is closely associated with vaccine coverage (Xu *et al*., 2015). The rapid increase in the frequency of Prn-deficient strains is perhaps the best evidence that *B. pertussis* are evolving away from vaccine-induced immunity. Furthermore, Prn deficient strains have shown better fitness in vaccinated mice (Hegerle *et al*., 2014; Safarchi *et al*., 2015), suggesting the evolution of Prn negative strains could improve the survival and transmission capability of *B. pertussis* in vaccinated populations.

However, only one Prn-deficient strain was identified in this collection, which could be due to the later introduction of the ACV in this country, the relatively low numbers of vaccine doses in the UK schedule compared to some other countries or that this vaccine is a 5 component, while others are 2-3 component, so the immune pressure is spread across more surface-associated antigens. The level of expression of Prn in outbreak and pre-outbreak strains was investigated, but no significant differences were observed to suggest that strains isolated during the outbreak had lower levels of Prn expression. Similarly, there were no significant differences between pre-outbreak and outbreak strains in their recognition by post-vaccine sera. However, this experiment should be repeated using a much larger panel of *B. pertussis* strains to gain a better perspective on the dynamics of immune recognition from strains isolated from different years. Unfortunately, due to time and funding, this was not possible.

Although further work is needed on the observation that *ptxP3* strains expressed significantly less Prn than *ptxP1* strains, work by Bouchez *et al* (2015) identified a

significant reduction in the transcription level of *prn* in *ptxP3* strains compared to *ptxP1* strains, adding weight to the observation seen in this thesis.

The finding that there was a positive correlation between Prn expression and recognition by post-vaccine sera suggests that anti- Prn IgG is a significant component of vaccine-induced responses. This is obviously desirable for a vaccine, but also increases selective pressure on this antigen to evolve towards evasion, which appears to have occurred in *B. pertussis*. The findings also highlight the effect Prn deficient strains have on the ability of post-vaccine sera to recognise and bind to the surface of *B. pertussis* strains, adding support that *prn* deficient strains could have survival advantage in a vaccinated population.

In conclusion, this project has provided evidence that the genes coding for vaccine antigen proteins are evolving to evade vaccine-induced immunity, and the introduction of the ACV has accelerated this affect, not only in the UK, but worldwide. This could have serious consequences for the ability of the current acellular vaccines to continue to control pertussis.

7

Future work

A number of interesting observations have been made in this project, some of which has produced preliminary work and providing a good basis to work on.

The data in Chapter 5 identified a positive correlation between Prn expression and recognition by post-vaccine sera. However adding a larger panel of strains to carry out this experiment would gain a better perspective on the dynamics of Prn expression and immune recognition. It would be worthwhile also including post-WCV-sera to see whether or not the same correlation is seen. It could be hypothesised that the positive correlation between post-WCV-sera and Prn expression would be weaker, due to the reduction in specificity of antibody recognition to antigens, supporting the view that the ACV has increased the selective pressure and therefore evolution of *B. pertussis* strains away from the vaccine type.

Follow up work on identifying differences in Prn expression between *ptxP1* and *ptxP3* strains would also be pursued as only two *ptxP1* strains were used in the current study. *PtxP3* strains are the dominant type circulating in worldwide and have shown increased fitness (King *et al*., 2013). Furthermore, Bouchez *et al* (2015b) identified a significant reduction of *prn* transcription in *ptxP3* strains compared to *ptxP1*. Understanding how this phenotype survives in a vaccinated population is important in recognising the reasons behind the resurgence seen.

Investigating other mechanisms of genetic variation in *B. pertussis* would be appropriate to pursue. The possibility of gene rearrangements playing a role in phenotypic plasticity could be likely, given that *B. pertussis* harbours ~240 copies of IS*481* which is known to play a major role in the evolution of *B. pertussis*. It is well documented that changes in the location of genes in the genome can affect the level of transcription, and expression profiles of genes (Sousa *et al*., 1997). Highly expressed genes are found on the leading strand of the genome (Price *et al*., 2005), but if these genes are then moved to the lagging strand, their expression is lowered. Similarly, if genes move further away from the origin of replication, for example those involved in Bvg modulation, this will also affect gene expression, which will lead to variation in phenotype. A single previous report identified

significant differences in transcript abundance between *B. pertussis* strains and also within the same strain as a result of genome rearrangements (Brinig *et al*., 2006).
In *B. pertussis* genome rearrangements occur by recombination between IS*481* elements (Parkhill *et al*., 2003). Resulting rearrangements of the genome could affect gene expression and thus the physiology of strains. Recently, a new sequencing system (Pacific Biosciences (PacBio)) has become available which produces long sequence reads (>5kb) that can span the IS*481* repeats in *B. pertussis* genomes. The Sanger institute performed PacBio sequencing on 5 clinical UK *B. pertussis* strains used in this thesis, which revealed a high level of genome rearrangements (Fig. 8.1) that were flanked by IS*481* elements. As part of the further work using the PacBio system, it would be worth further investigating genome rearrangements which could reveal a previously unrecognised variability among *B. pertussis*, which had been previously thought of largely as a single, homogenous entity.

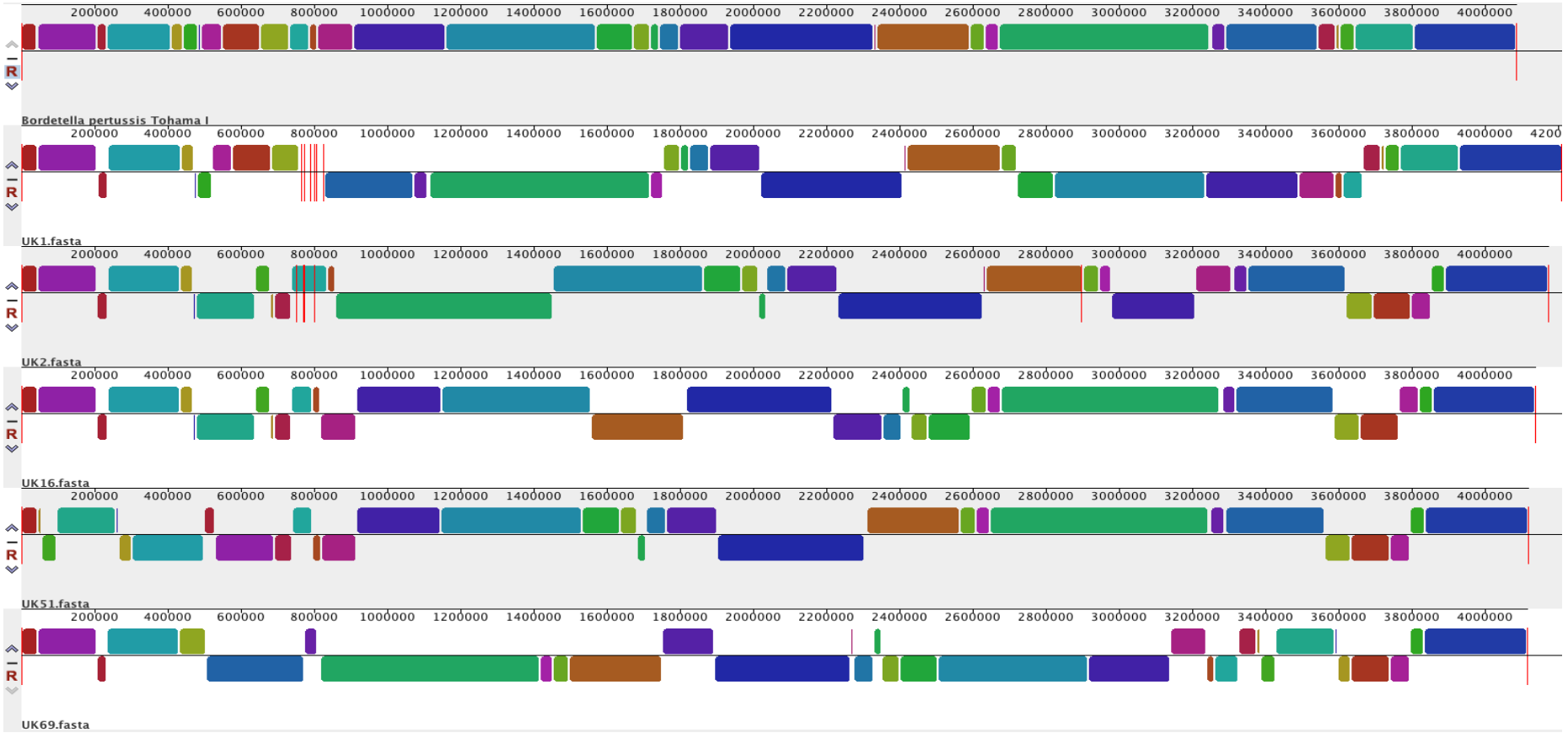

Fig.8.1. Alignment of the genome sequences of 6 *B. pertussis* strains reveals extensive variation in genome arrangement (A. Preston pers comm). Tohama I is the reference genome sequence produced by Sanger sequencing. The other five strains were sequenced using the PacBio platform. Assembly of data produced a single sequence contig for strains UK16, 51 and 69; but 5 contigs for UK 2 and 7 contigs for UK1 (shown by red lines in the alignment). Sequences were aligned using Mauve. The origin of replication is at coordinate 1 in each sequence. The different coloured segments are colinear regions of the genomes. Regions above the horizontal line are sequences of the plus DNA strand, below the line are on the minus strand. The relative position of colinear blocks, and the strand on which they located varies between strains indicating inversions and translocations have occurred within the genome of each strain.

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Appendix A

Metadata of Tohama I and the UK clinical strains used in this thesis.

Strain No.	PHE Identifier	Accession No.	Place of isolation/submission	Date of Isolation	Serotype	*ptxP*	*ptxA*	*prn*	*fim2*	*fim3*
Tohama I		BX470248	Japan	1954	1,2	1	2	1	1	1
UK01	NCTC10901	ERS018345	St Mary's Hospital, London	1920	1	1	2	1	1	1
UK02	CN0141	ERS018346	Hampstead, London	1942	1,2	1	2	1	1	1
UK03	CN1407	ERS018347	Oxford	1946	1,2,3	1	2	1	1	1
UK04	CN2055	ERS018348	LSHTM, London	1947	1,2	1	1	1	1	1
UK05	CN3001	ERS018349	CPHL, London	1949	1,3	1	2	1	1	1
UK06	NCTC10910	ERS018350	PHL, Coventry	1967	1,2,3	1	1	1	1	1
UK08	MANCH8002	ERS018349	PRL, Manchester	1979	1,2	1	1	1	1	1
UK09	DCH039	ERS018350	CAMR, Salisbury	1982	1,2	1	1	3	1	1
UK10	DCH088	ERS018353	CAMR, Salisbury	1982	1,2,3	1	1	1	1	1
UK11	DCH166	ERS018354	CAMR, Salisbury	1983	1,3	1	1	1	1	1
UK14	98K323	ERS018355	Aberystwyth	09-Nov-88	1,2	1	1	1	1	1
UK15	BP0865	ERS018356	Birmingham	02-Aug-02	1,3	3	1	2	1	2
UK16	H060580043	ERS018357	Slough	February 2006	1,2	1	1	1	1	1
UK17	H072360520	ERS018358	Manchester	01-Jun-07	1,3	3	1	2	1	2
UK18	H104520431	ERS018359	Stevenage	02-Nov-10	1,3	3	1	4	1	1
UK20	H094780268	ERS018360	Liverpool	15-Nov-09	1,3	3	1	2	1	3
UK21	H100760146	ERS018361	Manchester	February 2010	1,2	3	1	2	1	1
UK22	H100980734	ERS018362	Slough	March 2010	1,2	1	1	1	1	1

UK23	H102820210	ERS018363	Peterborough	28-Jun-10	1,3	3	1	2	1	1
UK24	H104460387	ERS018364	Kent	25-Oct-10	1,3	3	1		1	1
UK25	H080420101	ERS176852	Birmingham	09-Jan-08	1,3	3	1	2	1	1
UK26	H083360066	ERS176853	Manchester	28-Jul-08	1,3	3	1	2	1	1
UK27	H085180127	ERS176854	Leeds	22-Nov-08	1,3	3	1	2	1	2
UK28	H090760338	ERS176855	Cambridge	29-Jan-09	1,3	3	1	2	1	2
UK29	H094840822	ERS176856	Hounslow	21-Nov-09	1,2	3	1	2	1	1
UK30	H111420251	ERS176857	Derby	28-Mar-11	1,3	3	1	2	1	1
UK31	H111920356	ERS176858	Scunthorpe	27-Apr-11	1,2	1	1	3	1	1
UK32	H110620329	ERS176859	Southport	31-Jan-11	1,3	3	1	2	1	2
UK33	H111340193	ERS176860	Leicester	18-Mar-11	1,3	3	1	2	1	2
UK34	H113820371	ERS176861	Scarborough	19-Sep-11	1,2	3	1	2	1	1
UK35	H121080103	ERS176862	Kings Lynn	26-Feb-12	1,2	3	1	2	1	1
UK36	H121120872	ERS176863	Slough	06-Mar-12	1,3	3	1	2	1	1
UK37	H121120873	ERS176864	Slough	06-Mar-12	1,2	3	1	2	1	1
UK38	H121320680	ERS176865	Slough	05-Mar-12	1,3	3	1	2	1	2
UK39	H121340485	ERS176866	Southampton	14-Mar-12	1,3	3	1	2	1	2
UK40	H121340484	ERS176867	Leeds	18-Mar-12	1,2	3	1	2	1	1
UK41	H123080639	ERS176868	Halifax	20-Jul-12	1,2	3	1	2	1	1
UK42	H123120553	ERS176869	Ashford	11-Jul-12	1,3	3	1	2	1	1
UK43	H123140609	ERS176870	Norwich	20-Jul-12	1,3	3	1	2	1	1
UK44	H123180882	ERS176871	Truro	21-Jul-12	1,3	3	1	2	1	1
UK45	H123180902	ERS176872	Truro	19-Jul-12	1,3	3	1	2	1	1
UK46	H123040595	ERS176873	Cardiff	17-Jul-12	1,3	3	1	2	1	1
UK47	H123040596	ERS176874	Bristol	11-Jul-12	1,2	3	1	2	1	1
UK48	H123040597	ERS176875	Kingston	11-Jul-12	1,2	3	1	2	1	1
UK49	H123040834	ERS227750	Belfast	17-Jul-12	1,3	3	1	2	1	2

UK50	H123060621	ERS227751	Kettering	13-Jul-12	1,2	3	1		1	1
UK51	H115020415	ERS227752	Derby	01-Dec-11	1,3	1	1	1	1	1
UK52	H115060354	ERS227753	Plymouth	06-Dec-11	1,2	3	1	2	1	1
UK53	H120180199	ERS227754	Torbay	22-Dec-11	1,3	3	1	2	1	2
UK54	H120260403	ERS227755	Exeter	30-Dec-11	1,3	3	1	2	1	1
UK55	H120300223	ERS227756	Belfast	05-Jan-12	1,2	3	1		1	1
UK56	H120420417	ERS227757	St. Helier	13-Jan-12	1,2	1	1	1	1	1
UK57	H120520440	ERS227758	Newcastle-Upon-Tyne	17-Jan-12	1,3	3	1	2	1	2
UK58	H120620487	ERS227759	Exeter	28-Jan-12	1,2	3	1	2	1	1
UK59	H120700084	ERS227760	Poole	02-Feb-12	1,3	3	1	2	1	2
UK60	H120860175	ERS227761	Galway	21-Feb-12	1,2	3	1	2	1	1
UK61	H121000369	ERS227762	Dorchester	27-Feb-12	1,3	3	1	2	1	1
UK62	H121080104	ERS227763	Birmingham	02-Mar-12	1,2	3	1	2	1	1
UK63	H121120583	ERS227764	Ipswich	02-Mar-12	1,3	3	1		1	1
UK64	H121120585	ERS227765	Norwich	27-Feb-12	1,3	3	1		1	1
UK65	H121360522	ERS227766	Kettering	19-Mar-12	1,3	3	1	2	1	2
UK66	H121440482	ERS227767	Exeter	02-Apr-12	1,3	3	1	2	1	1
UK67	H121460713	ERS227768	Stockport	30-Mar-12	1,2	3	1	2	1	1
UK68	H121560437	ERS227769	Frimley	03-Apr-12	1,2,3	3	1	2	1	1
UK69	H121560438	ERS227770	Worthing	03-Apr-12	1,3	3	1	2	1	2
UK70	H121940701	ERS227771	Burton-On-Trent	01-May-12	1,3	3	1	2	1	1
UK71	H121940704	ERS227772	Cardiff	20-Apr-12	1,2	3	1	2	1	1
UK72	H122040731	ERS227773	Dorchester	10-May-12	1,3	3	1	2	1	1
UK73	H122120592	ERS227774	Nottingham	10-May-12	1,2	3	1	2	1	1
UK74	H122220497	ERS227775	Southport	29-May-12	1,3	3	1	2	1	2
UK75	H122380455	ERS227776	Leicester	01-Jun-12	1,3	3	1	2	1	2
UK76	H122560437	ERS227777	Bristol	06-Jun-12	1,2	3	1	2	1	1

UK77	H122580435	ERS227778	North Shields	21-Jun-12	1,3	3	1	2	1	1
UK78	H122700116	ERS227779	Bristol	14-Jun-12	1,2	3	1	2	1	1
UK79	H122860244	ERS227780	Stoke-On-Trent	02-Jul-12	1,2	3	1	2	1	1
UK80	H122920208	ERS227781	Ipswich	06-Jul-12	1,3	3	1		1	2
UK81	H122980531	ERS227782	Boston	09-Jul-12	1,3	3	1	2	1	1
UK82	H123000341	ERS227783	Newcastle-Upon-Tyne	11-Jul-12	1,3	3	1	2	1	1
UK83	H123300481	ERS227784	Nottingham	01-Aug-12	1,2	3	1	2	1	1
UK84	H123380565	ERS227785	Ipswich	06-Aug-12	1,2	3	1	2	1	1
UK85	H123600404	ERS227786	York	23-Aug-12	1,3	3	1	2	1	1
UK86	H123620609	ERS227787	Scunthorpe	03-Sep-12	1,2	3	1		1	1
UK87	H123760736	ERS227788	Birmingham	28-Aug-12	1,3	3	1		1	1
UK88	H123800680	ERS227789	Sheffield	05-Sep-12	1,3	3	1	3	1	2
UK89	H123880654	ERS227790	Oldham	12-Sep-12	1,3	3	1	2	1	2
UK90	H123940665	ERS227791	Crawley	13-Sep-12	1,3	3	1	2	1	2
UK91	H124140572	ERS227792	Dorchester	01-Oct-12	1,3	3	1	2	1	1
UK92	H124320838	ERS227793	Bristol	03-Oct-12	1,2	3	1	2	1	1
UK93	H124620975	ERS227794	Newcastle-Upon-Tyne	04-Nov-12	1,3	3	1	2	1	2
UK94	H124740775	ERS227795	Durham	12-Nov-12	1,2	3	1	2	1	1
UK95	H124960781	ERS227796	Leeds	28-Nov-12	1,3	3	1	2	1	2
UK96	H125040728	ERS227797	Gloucester	30-Nov-12	1,2					
UK97	H125080782	ERS227798	Halifax	03-Dec-12	1,3	3	1	2	1	1
UK98	H125180676	ERS227799	Truro	10-Dec-12	1,3	3	1	2	1	2
UK99	H125180677	ERS227800	Bristol	12-Dec-12	1,2	3	1	2	1	1
UK100	H130220686	ERS227801	Portsmouth	28-Dec-12	1,3	3	1	2	1	2

Strain No.	Age	Sex	Vaccinated	No. doses	Date of onset	Macrolide given	Apnoeic attacks	Convulsions	Pneumonia	Death	Admitted to Hospital
Tohama I	NK	NK	NK		NK	NK	NK	NK	NK	NK	NK
UK01	NK	NK	NK		NK	NK	NK	NK	NK	NK	NK
UK02	NK	NK	NK		NK	NK	NK	NK	NK	NK	NK
UK03	NK	NK	NK		NK	NK	NK	NK	NK	NK	NK
UK04	NK	NK	NK		NK	NK	NK	NK	NK	NK	NK
UK05	NK	NK	NK		NK	NK	NK	NK	NK	NK	NK
UK06	NK	NK	NK		NK	NK	NK	NK	NK	NK	NK
UK08	NK	NK	NK		NK	NK	NK	NK	NK	NK	NK
UK09	NK	NK	NK		NK	NK	NK	NK	NK	NK	NK
UK10	NK	NK	NK		NK	NK	NK	NK	NK	NK	NK
UK11	NK	NK	NK		NK	NK	NK	NK	NK	NK	NK
UK14	20 m	M	YES	NK	NK	NK	NK	NK	NK	NK	NK
UK15	1 y 8 m	M	NK		NK	NK	NK	NK	NK	NK	NK
UK16	6 w	M	NO		January 2006	Yes	YES	NO	YES	NO	YES
UK17	1 m	M	NO		NK	NK	NK	NK	NK	NK	YES
UK18	1 m	M	YES	1	28/10/10	NK	YES	NO	NO	NO	YES
UK20	2 m	F	YES	3	07/12/09	YES	NO	NO	NO	NO	NO
UK21	1 m	F	NK		NK	NK	NK	NK	NK	NK	NK
UK22	1 m	M	NO		21/02/10	YES	YES	NO	YES	NO	YES

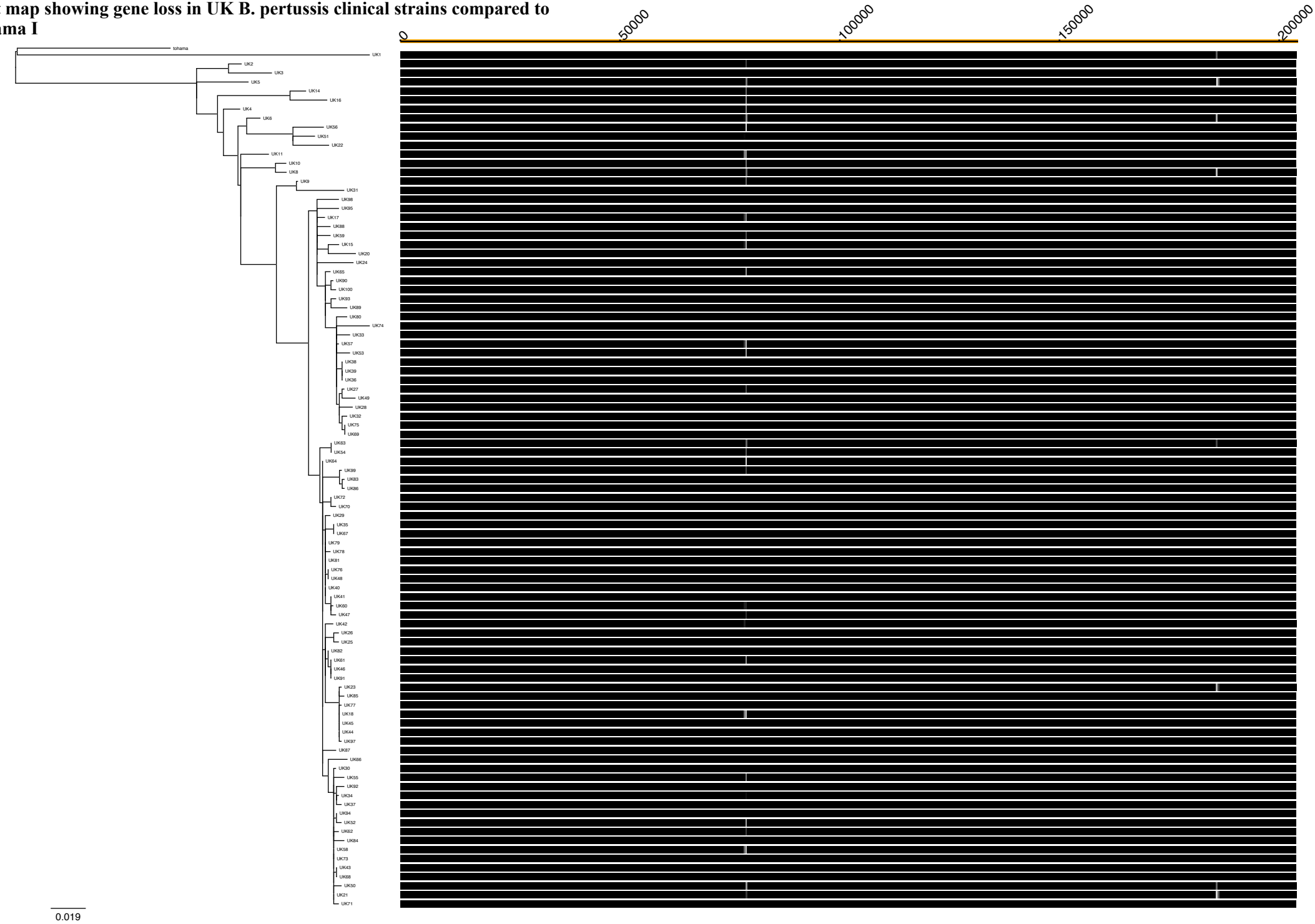
UK23	3 w	F	NO		21/06/10	YES	YES	NO	NK	NO	YES
UK24	5 d	F	No		00/01/1900	NK	NK	NK	NK	NK	NK
UK25	3 m	F	YES	1	05/01/08	YES	NO	NO	NO	NO	YES
UK26	2 m	U	NO		15/07/08	YES	NO	NO	NO	NO	NK
UK27	2 m	M	NO		18/11/08	NO	NO	NO	NO	NO	YES
UK28	3 m	F	YES	2	01/01/09	YES	NO	NO	NO	NO	NO
UK29	2 m	M	NO		MidNovember 2009	YES	NO	NO	NO	YES	YES
UK30	14 y	F	YES	3	14/03/11	YES	NO	NO	NO	NO	NO
UK31	52 y	F	NO		07/04/11	YES	NO	NO	NO	NO	NO
UK32	1m	F	NO		20/01/11	YES	NO	NO	NK	NO	YES
UK33	1m	M	NK		08/03/11	NK	NO	NO	YES	YES	YES
UK34	3m	M	NO		30/08/11	YES	NO	NO	NO	NO	YES
UK35	1m	M	NO		NK	NO	YES	NK	YES	YES	YES
UK36	15y	M	YES	3	01/03/12	YES	NO	NO	NO	NO	NO
UK37	13y	M	YES	4	04/03/12	YES	NO	NO	NO	NO	NO
UK38	14y	M	YES	3	28/02/12	YES	NO	NO	NO	NO	NO
UK39	16y	M	YES	3	09/03/12	YES	NO	NO	NO	NO	NO
UK40	2m	M	NO		14/03/12	NK	NK	NK	NK	YES	YES
UK41	1m	M	NK		NK	NK	NK	NK	NK	NK	YES
UK42	2m	F	NO		28/06/12	YES	NK	NO	NO	NO	YES
UK43	1m	M	NO		10/07/12	YES	NK	NK	NO	NK	YES
UK44	8m	M	YES	1	11/07/12	NK	NK	NK	NK	NK	YES
UK45	8m	F	NK		11/07/12	NO	NK	NK	NK	NK	YES
UK46	1m	M	NO		14/07/12	YES	YES	NK	NK	NO	YES
UK47	2m	F	NO		02/07/12	NO	NO	NO	YES	NO	YES
UK48	1m	F	NO		16/06/12	YES	NO	NO	NK	NO	YES
UK49	2m	M	NK		NK	NK	NK	NK	NK	NK	YES

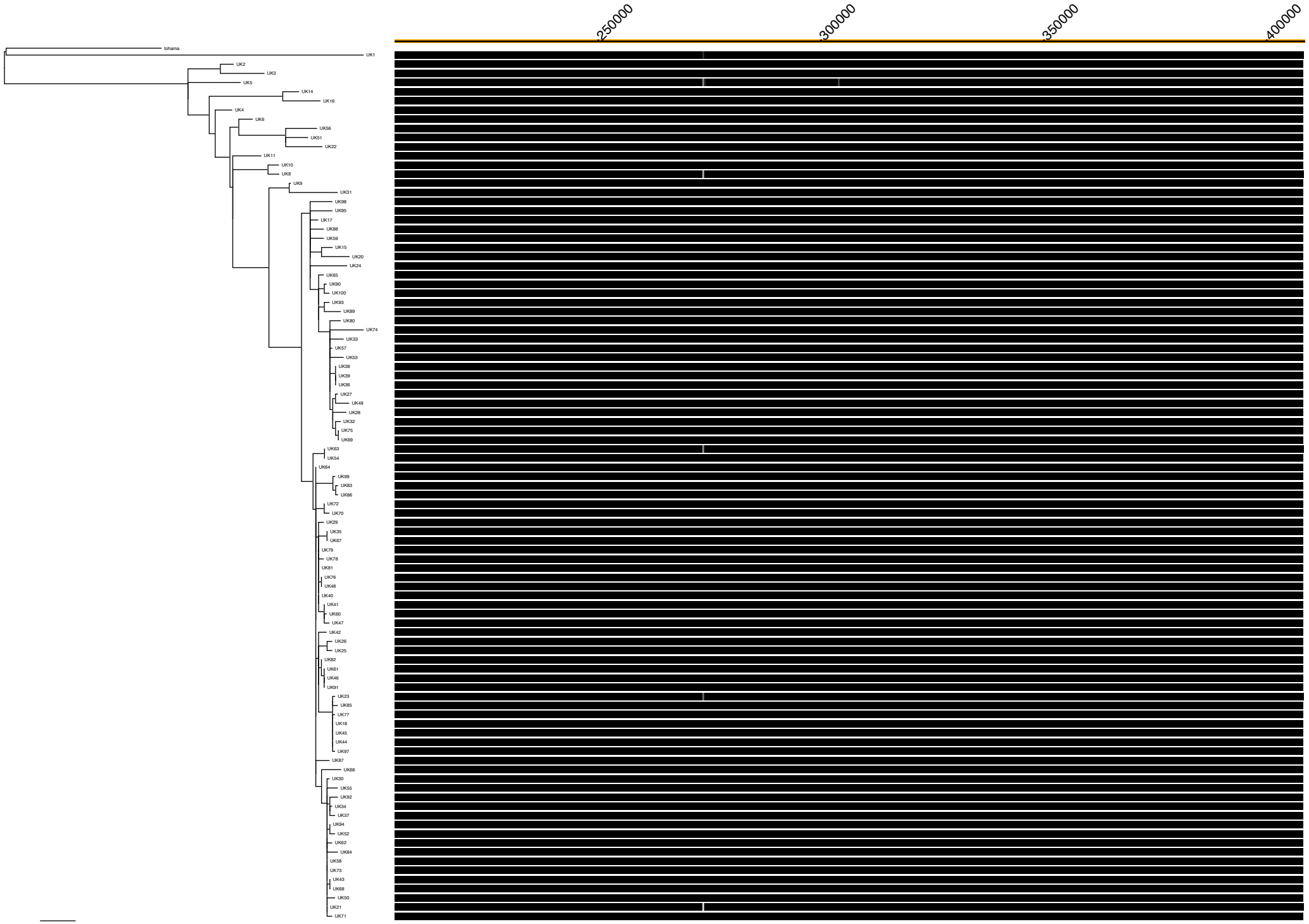
UK50	1m	M	NO		02/07/12	NO	YES	NO	NO	NO	YES
UK51	8m	M	NO		22/11/11	NO	NO	NO	NO	NO	YES
UK52	1m	M	NO		23/11/11	YES	YES	NO	NK	NO	YES
UK53	11 y 6m	M	NO		NK	YES	NO	NO	NO	NO	NO
UK54	16 y 2m	F	YES	3	NK	NK	NK	NK	NK	NK	YES
UK55	2 m	M	NK		NK	NK	NK	NK	NK	NK	YES
UK56	22 y 3 m	F	NK		NK	NK	NK	NK	NK	NK	NK
UK57	3m	F	YES	2	17/01/12	YES	NK	NK	NK	NK	YES
UK58	1m	M	NO		16/01/12	NK	NO	NO	NO	NO	YES
UK59	1m	M	NO		23/01/12	NK	NO	NO	NO	NO	YES
UK60	4y 4m	F	NK		NK	NK	NK	NK	NK	NK	YES
UK61	12y 1m	M	YES	4	ca.January 2012	YES	NO	NO	NO	NO	YES
UK62	1m	M	NO		28/02/12	YES	NO	NO	NO	NO	YES
UK63	64y 6m	M	NK		22/02/12	YES	NO	NO	NO	NO	NO
UK64	10m	M	NK		06/02/12	YES	NK	NK	NK	NK	YES
UK65	12y 2m	F	NO		07/03/12	YES	NO	NO	NO	NO	NO
UK66	1m	F	NO		21/03/12	YES	NO	NO	NO	NO	YES
UK67	3m	M	NO		14/03/12	YES	NO	NO	YES	NO	YES
UK68	2m	F	NO		26/03/12	YES	NO	NO	NO	NO	YES
UK69	14y 10m	F	YES	3	14/02/12	YES	NO	NO	NO	NO	NO
UK70	2m	M	YES	1	15/04/12	YES	NK	NO	NO	NO	YES
UK71	2m	F	NO		01/04/12	YES	NK	NK	NK	NO	YES
UK72	27y 3m	M	YES		08/05/12	YES	NO	NO	NO	NO	NO
UK73	1 m	M	NK		NK	NK	NK	NK	NK	NK	YES
UK74	4m	F	YES	2	08/05/12	YES	NK	NK	NO	NO	YES
UK75	3m	F	NO		28/04/12	YES	NO	NO	NO	NO	YES
UK76	59y 2m	F	NK		01/05/12	YES	NO	NO	YES	NO	NO

UK77	1y 5m	F	YES	3	15/05/12	YES	NO	NO	NO	NO	NO
UK78	28y 9m	F	NK		01/06/12	YES	NO	NO	NO	NO	NO
UK79	4m	F	NO		02/06/12	YES	NK	NO	NO	NO	YES
UK80	55y 10m	M	NO		05/07/12	YES	NO	NO	NO	NO	NO
UK81	2y	M	NO		29/06/12	YES	NO	NO	NO	NO	NO
UK82	29y 11m	M	YES	3	02/07/12	YES	NK	NK	NK	NK	NO
UK83	10m	F	NO		23/07/12	NO	NO	NO	NO	NO	NO
UK84	48 y 7 m	F	YES	3	Mid-July 2012	YES	NO	NO	NO	NO	NO
UK85	15 y 3 m	M	YES	3	01/08/12	YES	NO	NO	NO	NO	YES
UK86	6 m	M	NO		24/08/12	YES	NO	NO	NO	NO	NO
UK87	1 y 11 m	F	YES	3	07/08/12	NO	NO	NO	NO	NO	YES
UK88	50 y 10 m	M	NO		28/08/12	YES	NO	NO	NO	NO	YES
UK89	8 y 3 m	M	YES	3	07/09/12	YES	NO	NO	NO	NO	NO
UK90	5 y 2m	F	NO		06/09/12	NO	NO	NO	NO	NO	NO
UK91	2 m	F	NO		24/09/12	NO	YES	NO	NO	NO	YES
UK92	70 y 11 m	F	NK		01/10/12	YES	NO	NO	NO	NO	NO
UK93	3 w	M	NO		NK	NK	NK	NK	NK	NO	YES
UK94	45 y 10 m	F	NK		30/10/12	YES	NO	NO	NO	NO	NO
UK95	81 y 7 m	F	NO		19/11/12	YES	NO	NO	NO	NO	NO
UK96	28 y 7m	F	YES	3	NK	YES	NO	NO	NO	NO	NO
UK97	1 m	M	NO		03/12/12	YES	NK	NK	NK	NO	YES
UK98	46 y 5m	F	NK		03/12/12	YES	NO	NO	NO	NO	NO
UK99	24 y 4m	M	YES	3	05/12/12	YES	NO	NO	NO	NO	NO
UK100	13 y 5 m	F	NK		NK	NK	NK	NK	NK	NO	NO

Appendix B

Heat map showing gene loss in UK B. pertussis clinical strains compared to Tohama I

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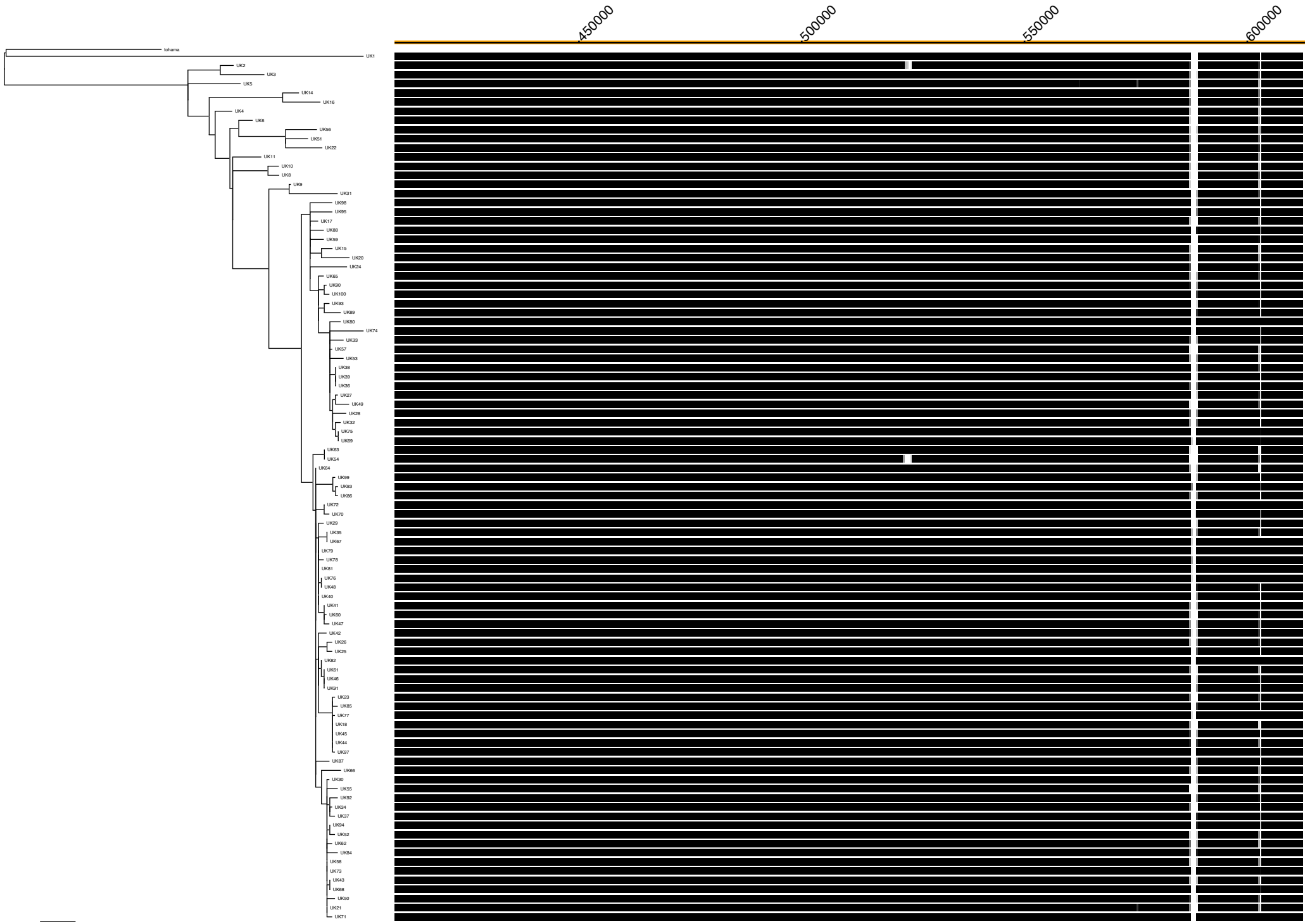
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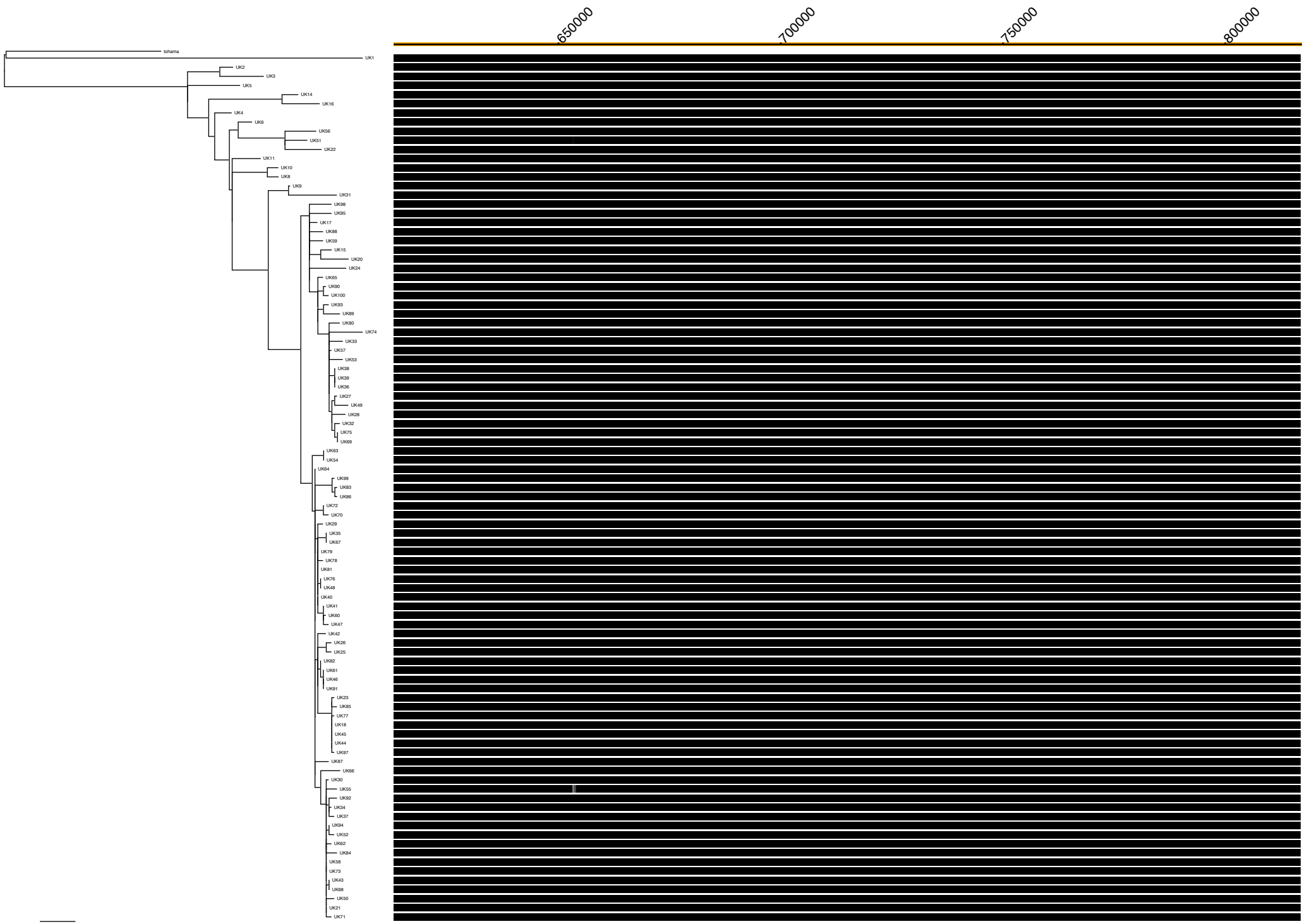
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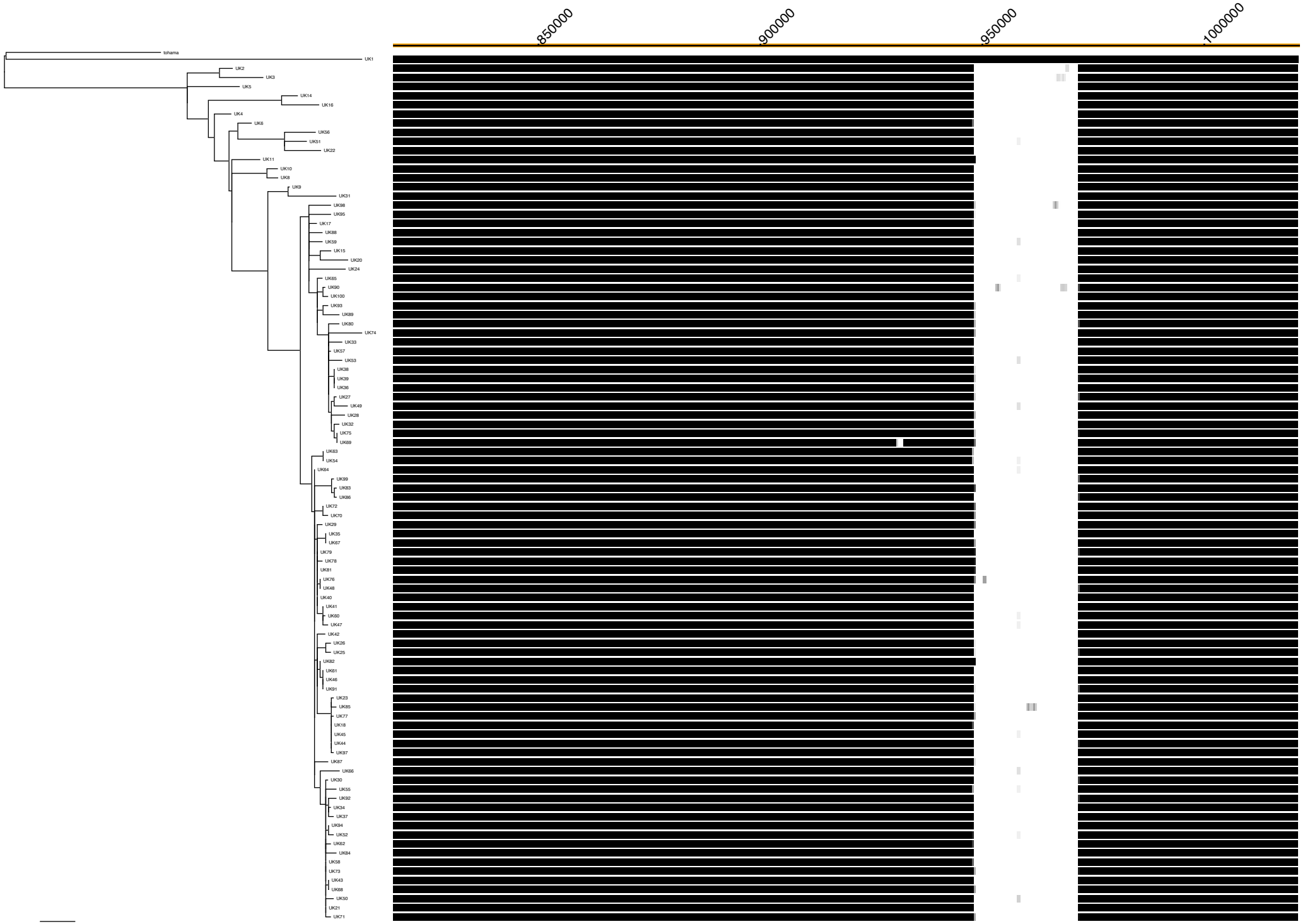
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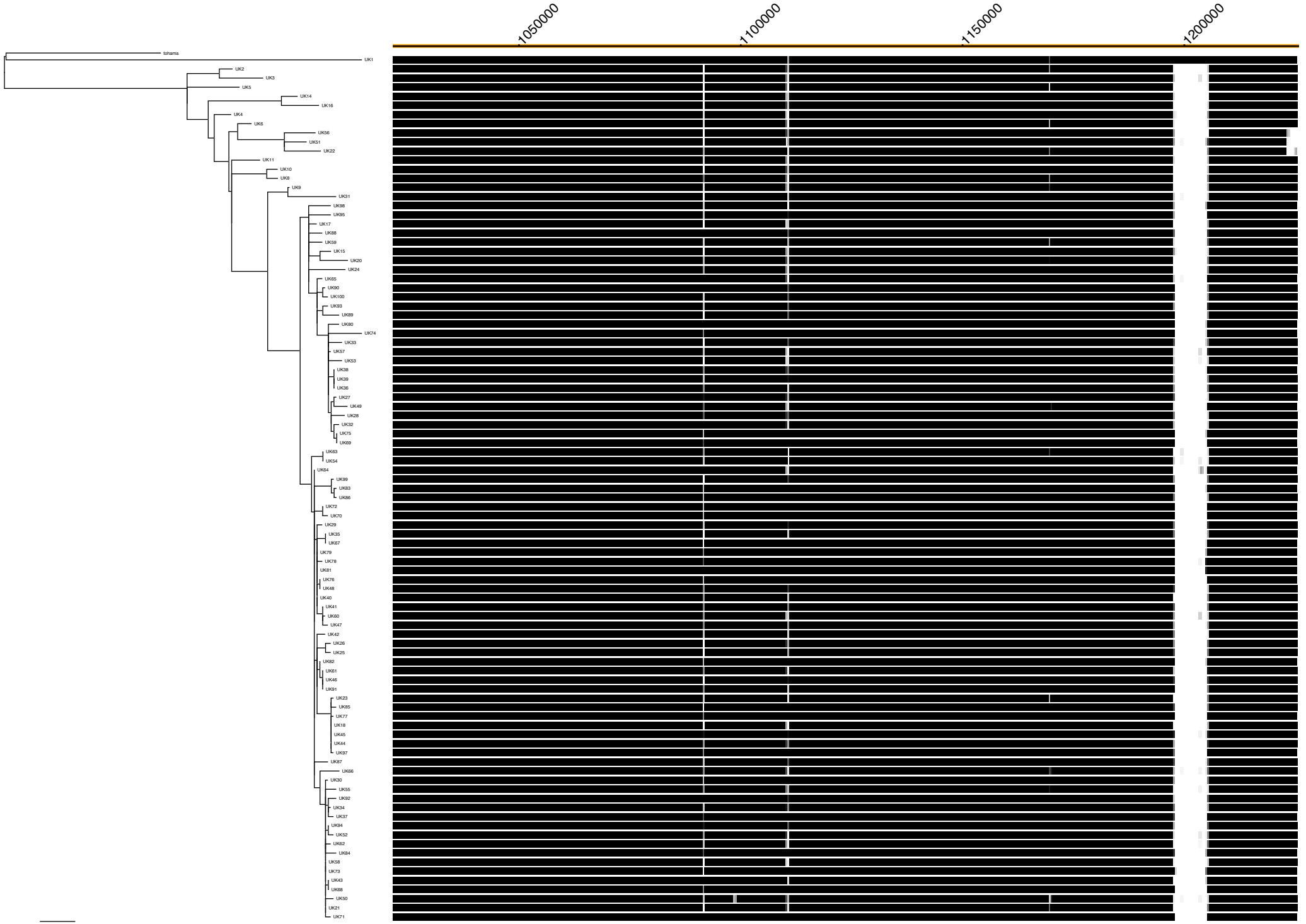
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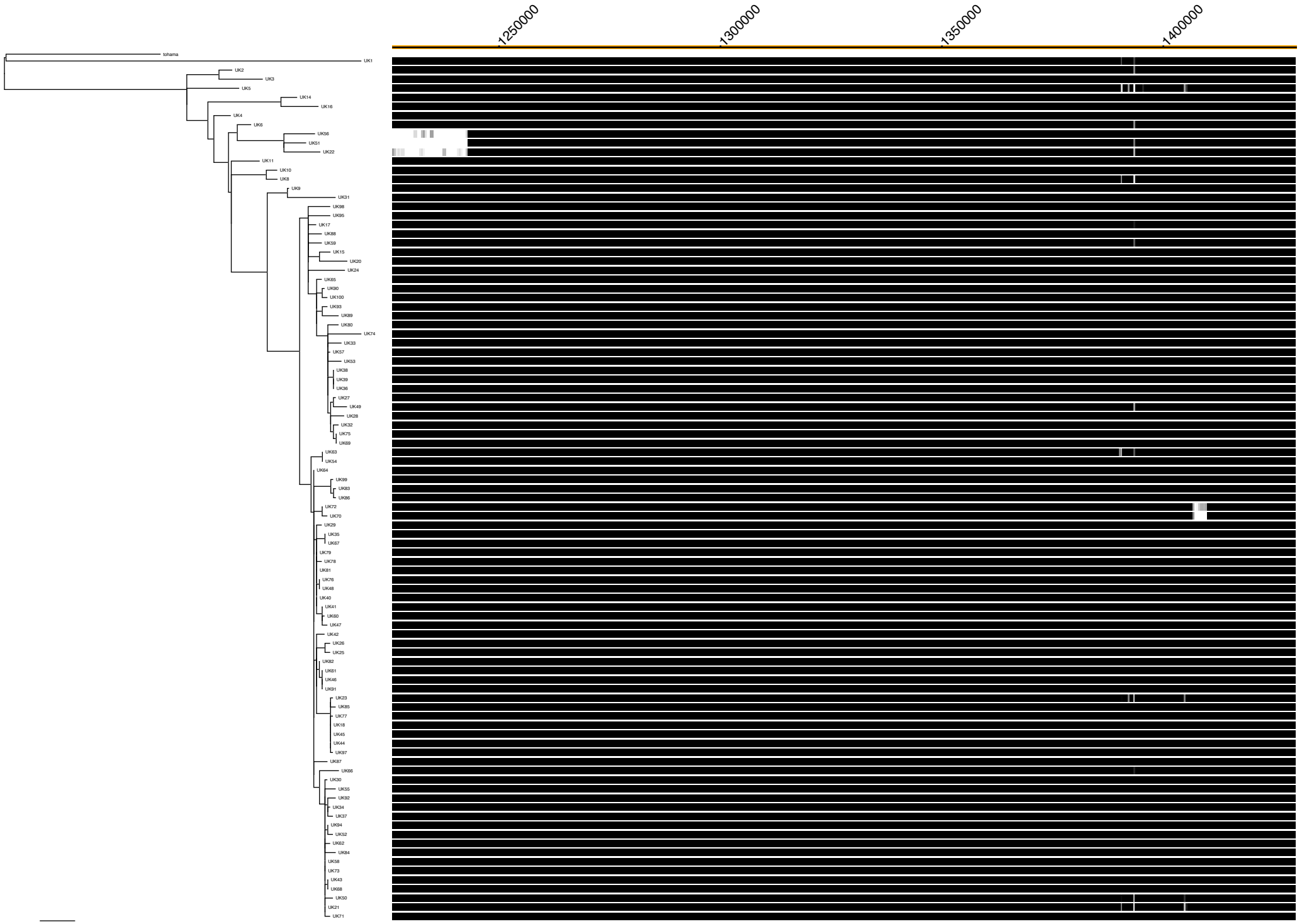
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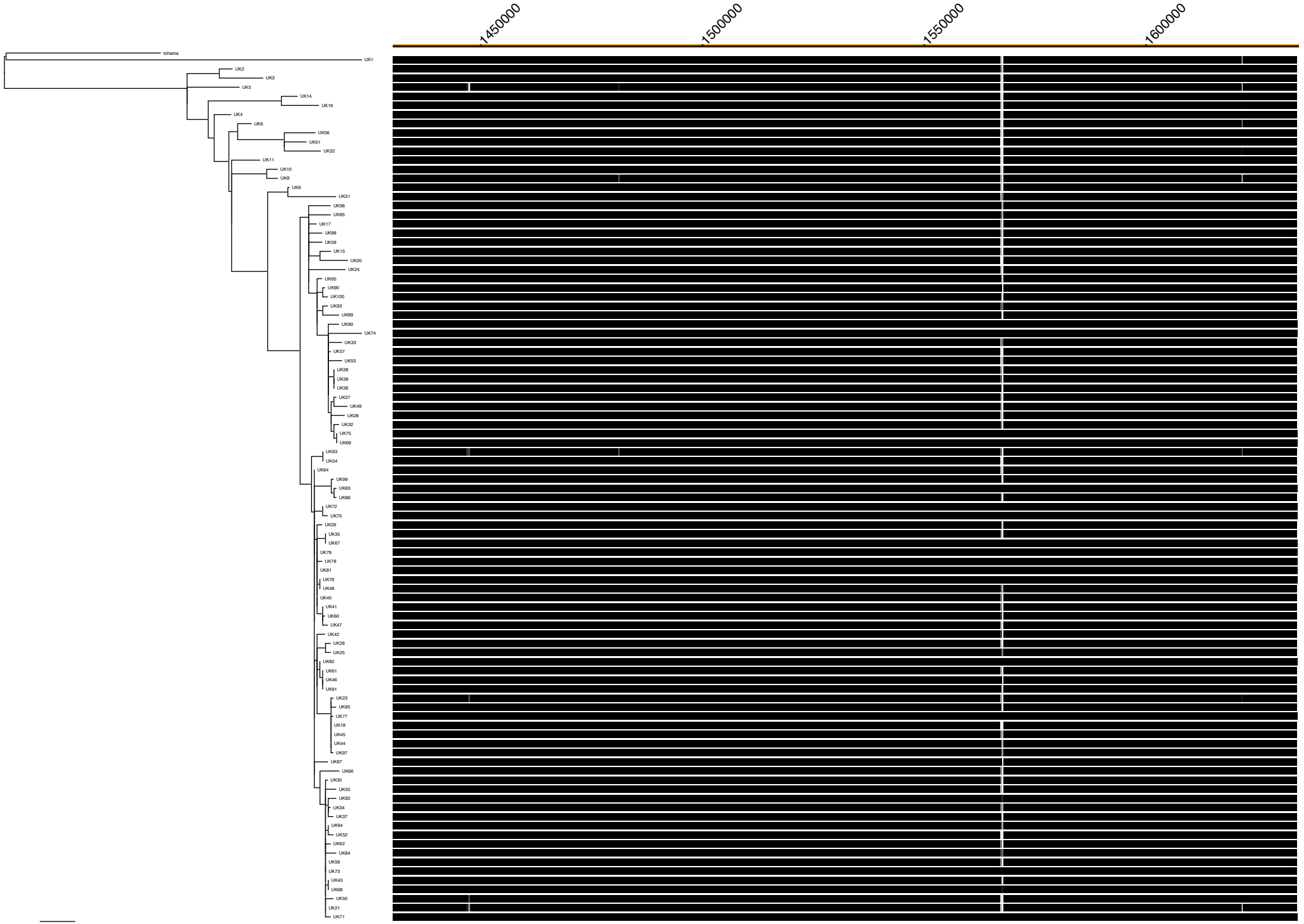
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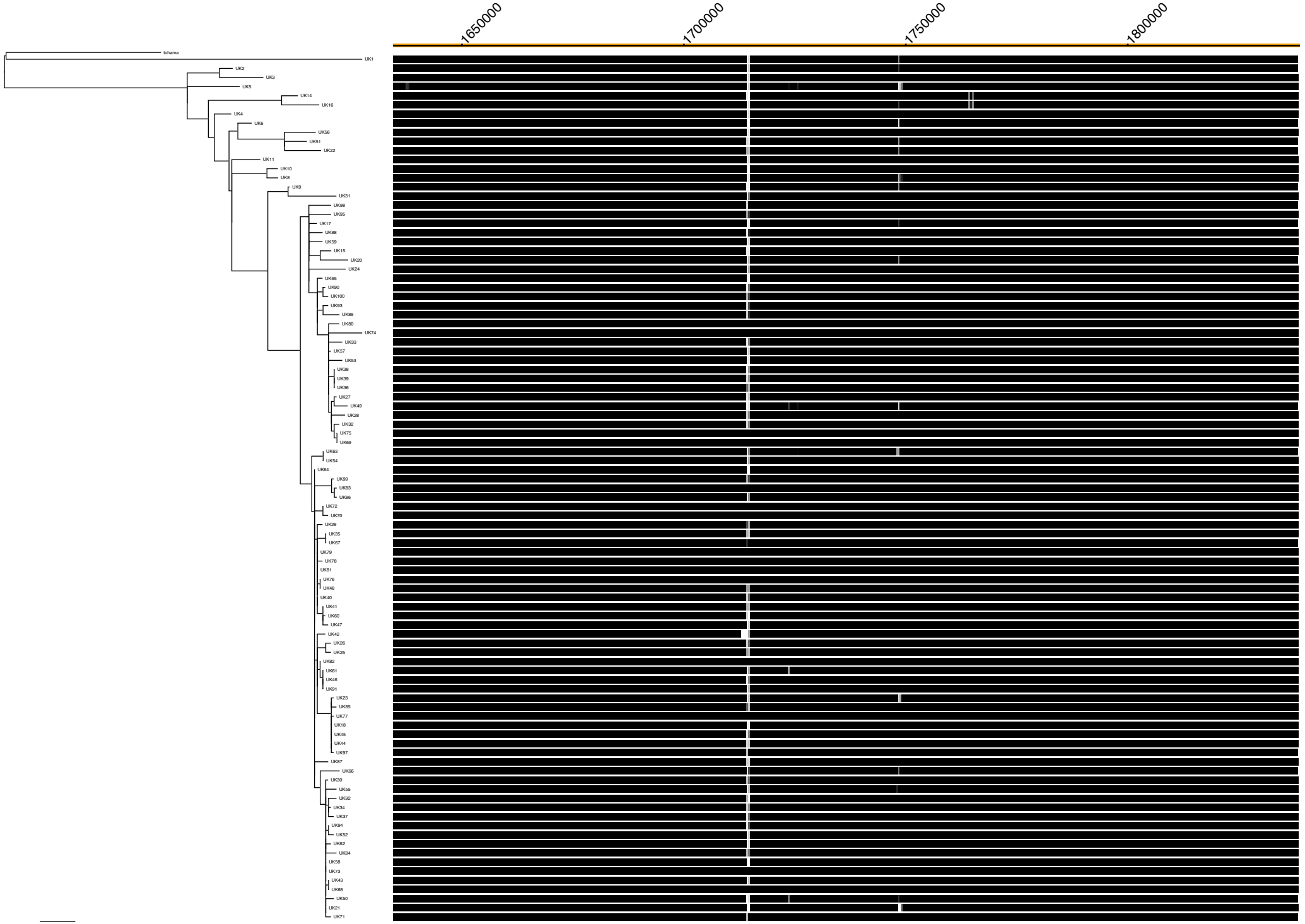
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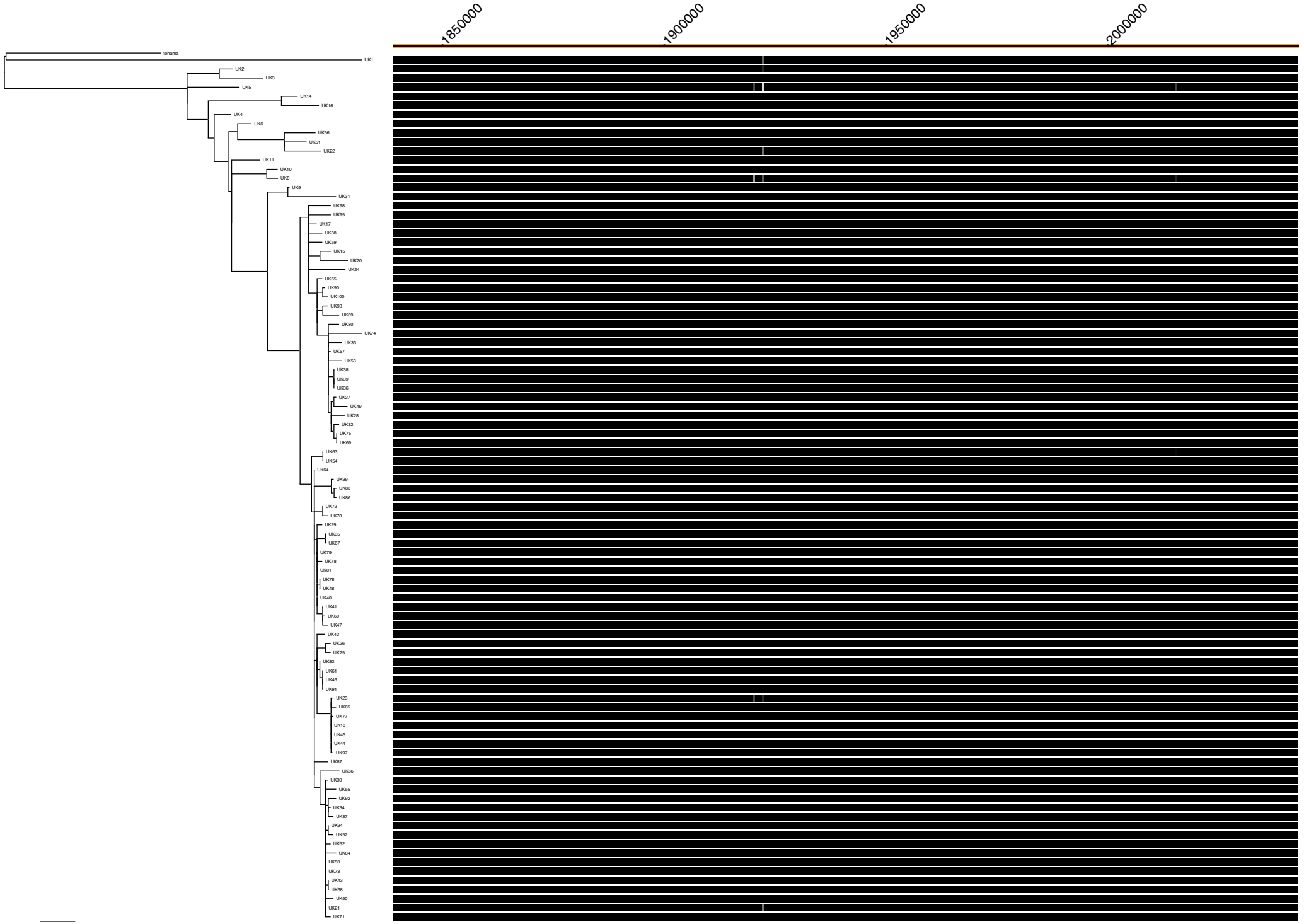
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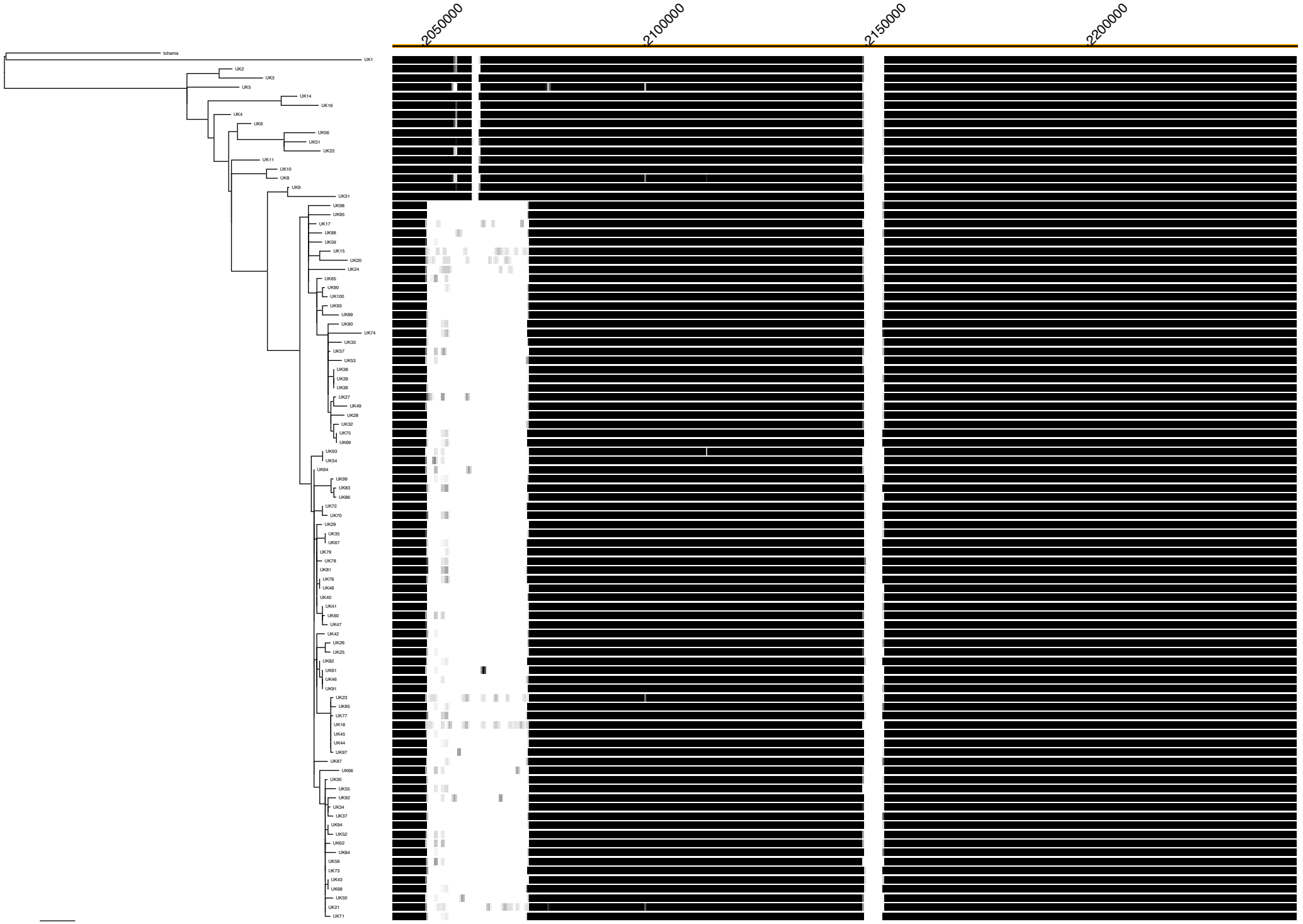
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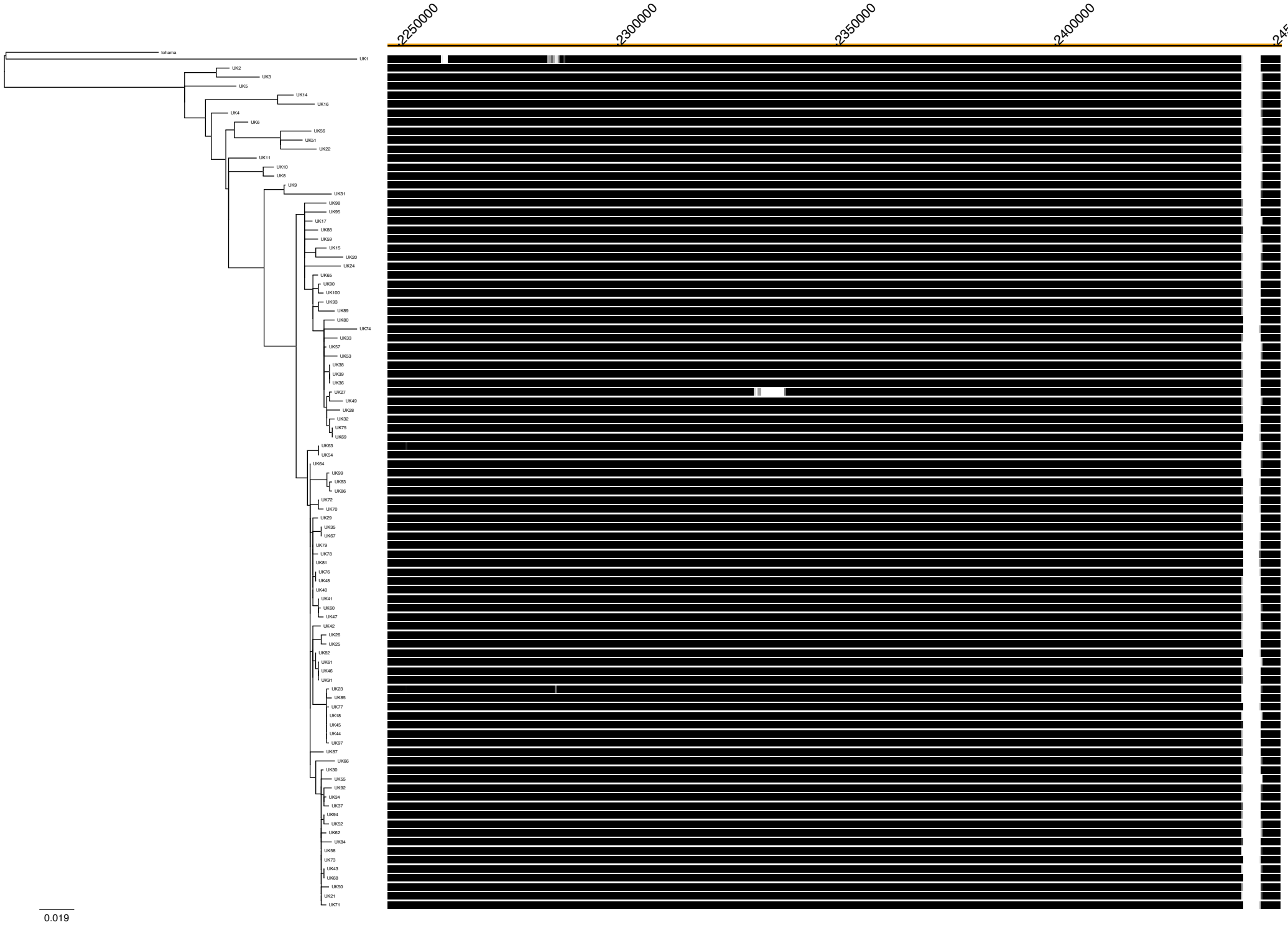
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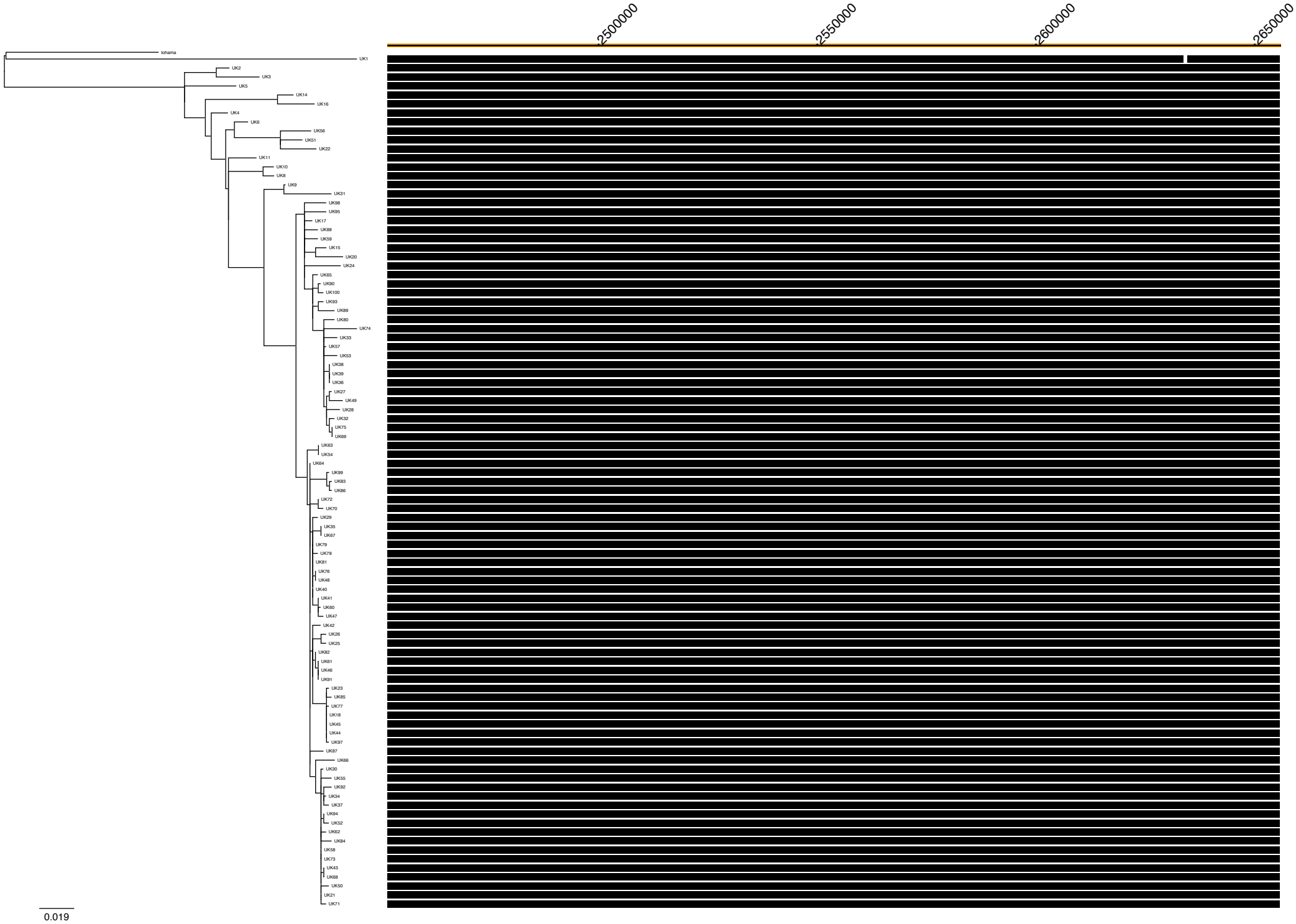
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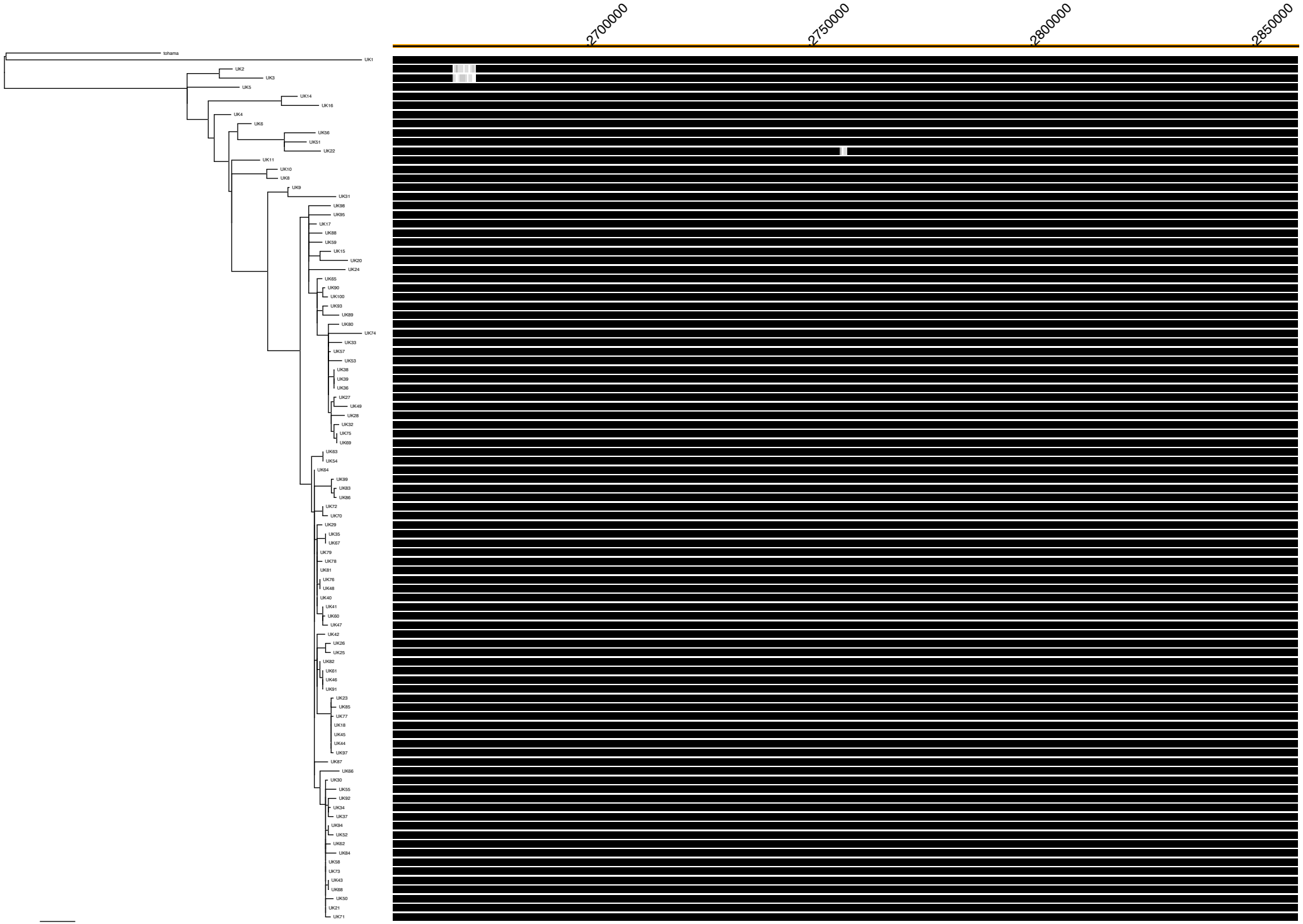
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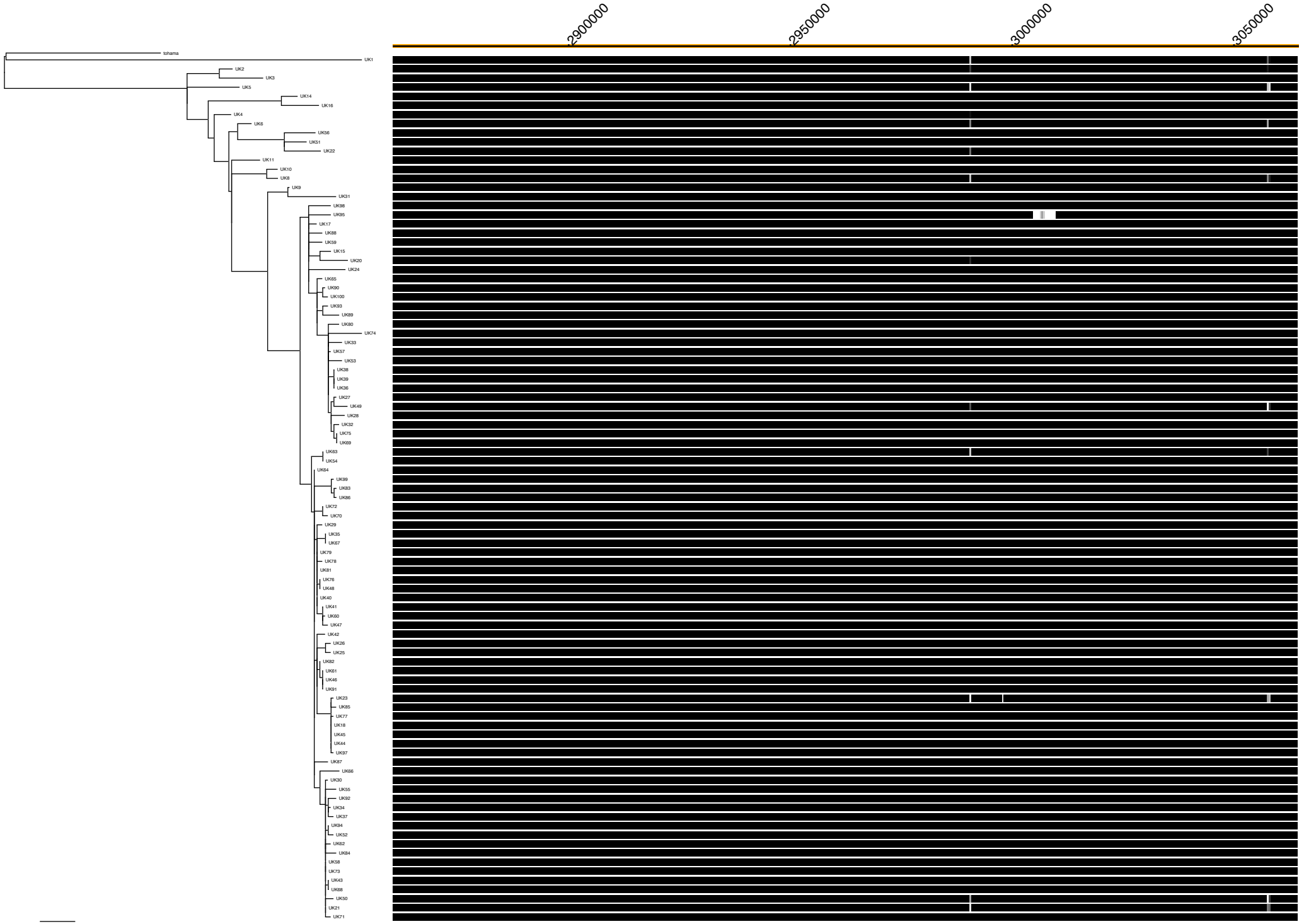
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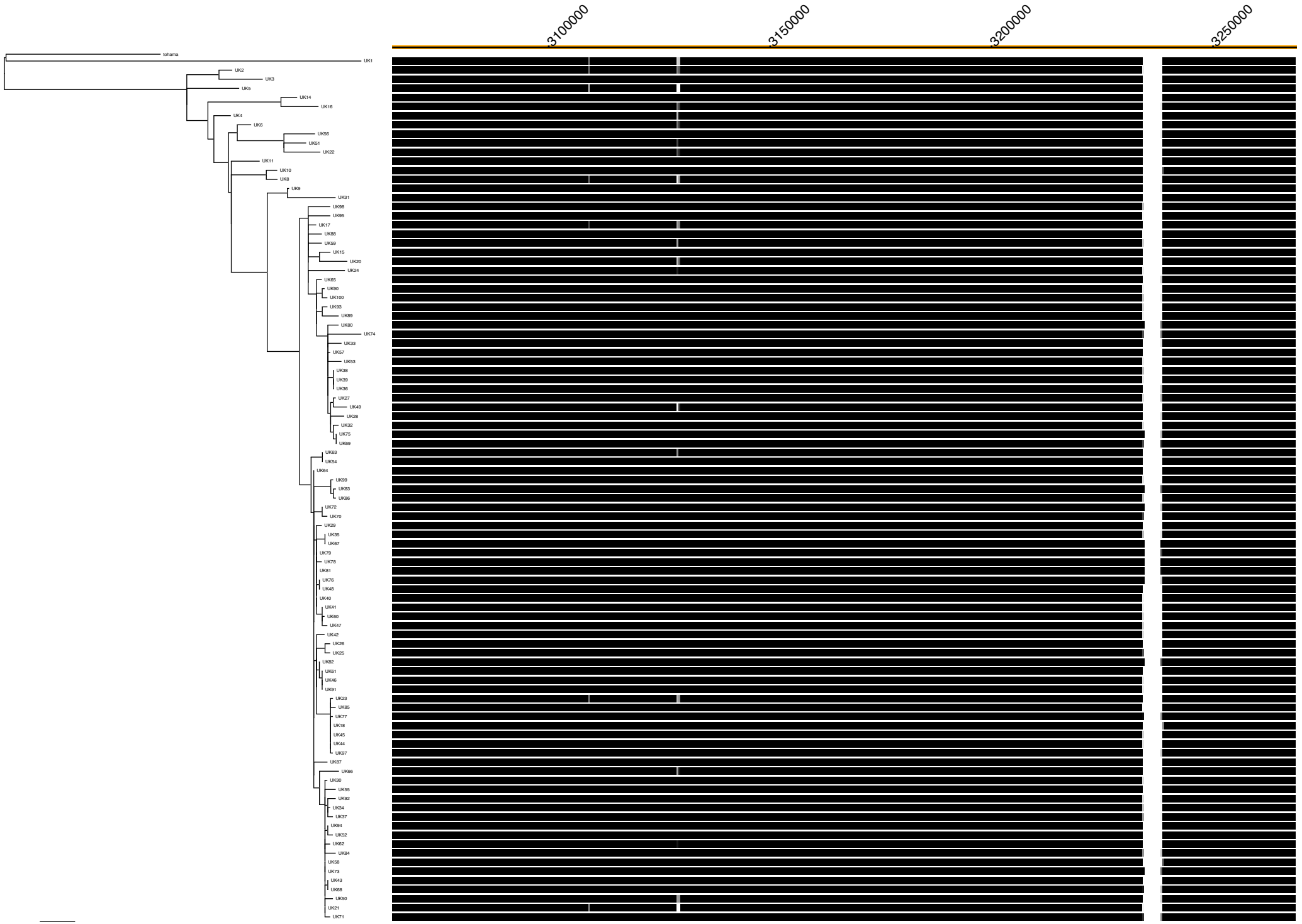
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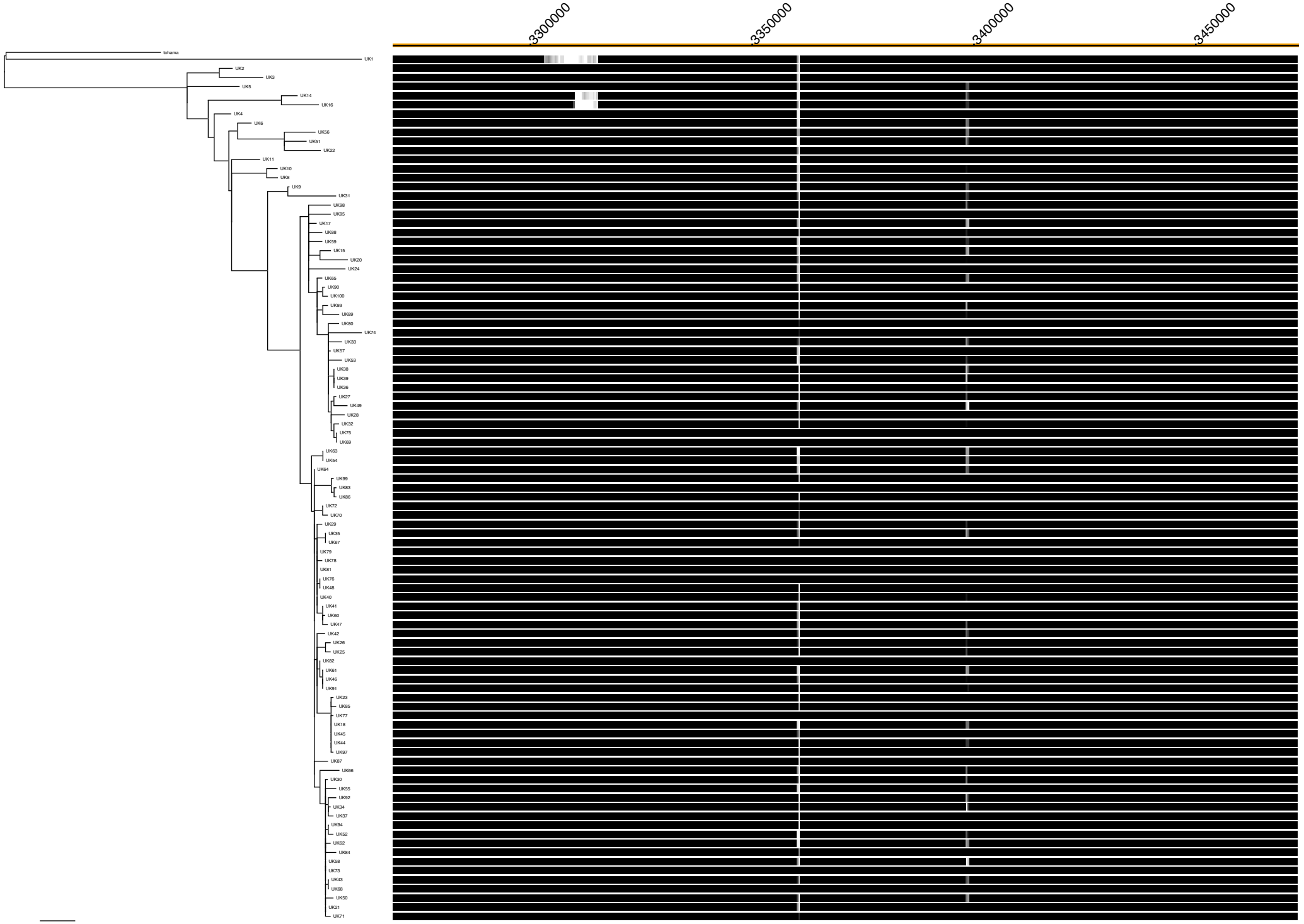
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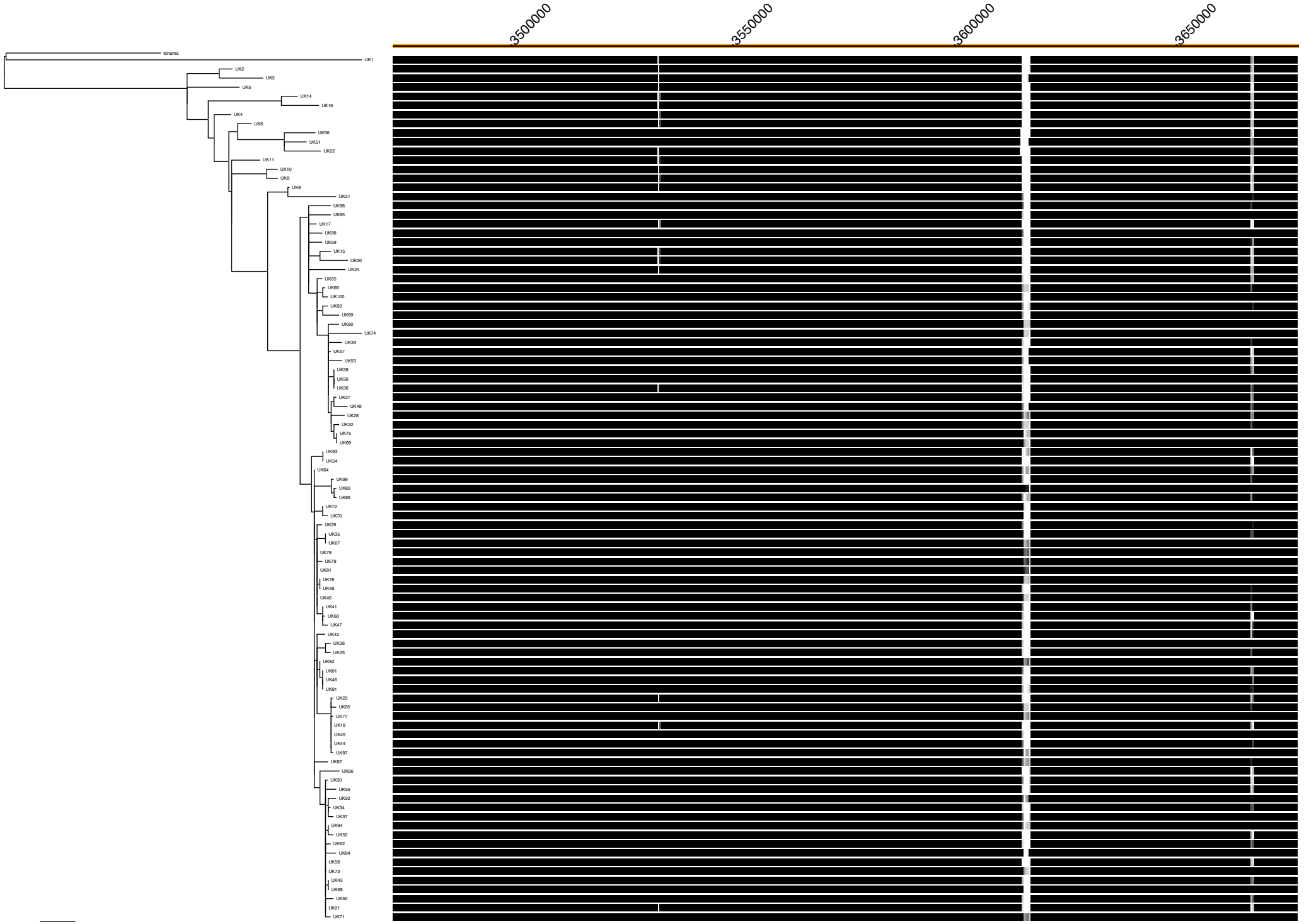
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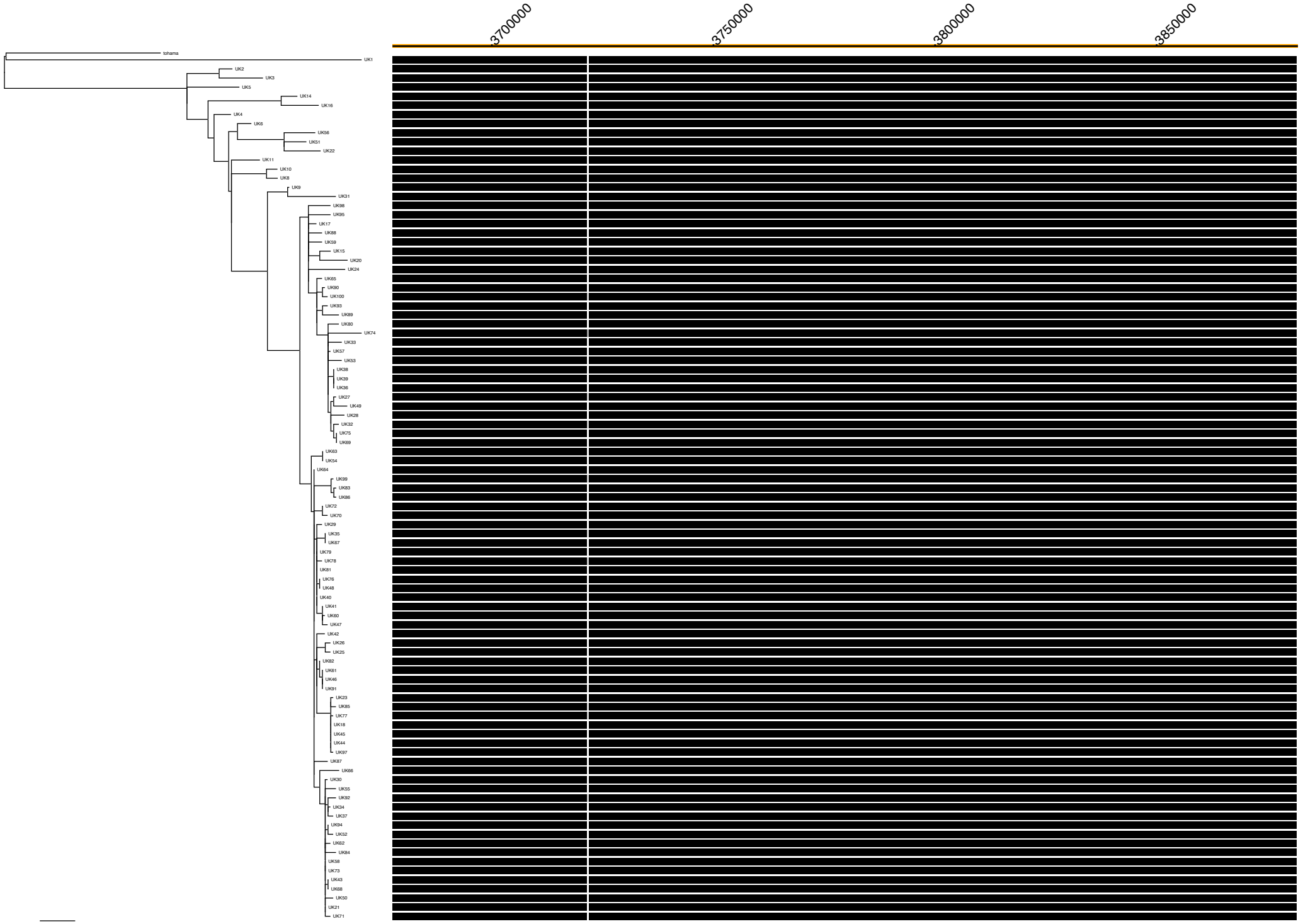
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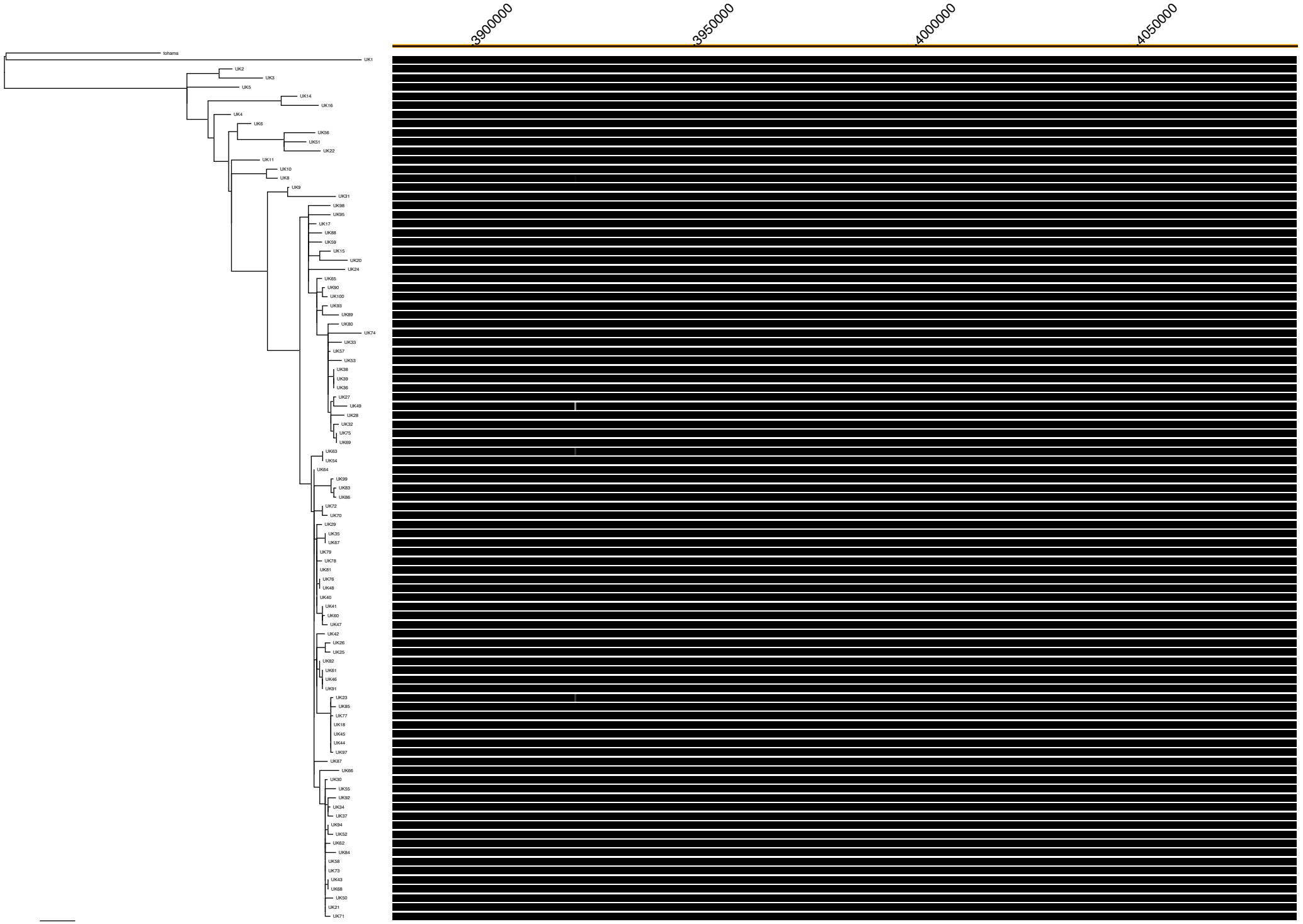
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MAJOR ARTICLE

Genomic Analysis of Isolates From the United Kingdom 2012 Pertussis Outbreak Reveals That Vaccine Antigen Genes Are Unusually Fast Evolving

Katie L. Sealey,[1,2] Simon R. Harris,[3] Norman K. Fry,[2] Laurence D. Hurst,[1] Andrew R. Gorringe,[4] Julian Parkhill,[3] and Andrew Preston[1]

[1]Department of Biology and Biochemistry, University of Bath, [2]Public Health England, Respiratory and Vaccine Preventable Bacteria Reference Unit, London, [3]Wellcome Trust Sanger Institute, Hinxton, and [4]Public Health England, Porton Down, Salisbury, United Kingdom

A major outbreak of whooping cough, or pertussis, occurred in 2012 in the United Kingdom (UK), with nearly 10 000 laboratory-confirmed cases and 14 infant deaths attributed to pertussis. A worldwide resurgence of pertussis has been linked to switch to the use of acellular pertussis vaccines and the evolution of *Bordetella pertussis* away from vaccine-mediated immunity. We have conducted genomic analyses of multiple strains from the UK outbreak. We show that the UK outbreak was polyclonal in nature, caused by multiple distinct but closely related strains. Importantly, we demonstrate that acellular vaccine antigen–encoding genes are evolving at higher rates than other surface protein–encoding genes. This was true even prior to the introduction of pertussis vaccines but has become more pronounced since the introduction of the current acellular vaccines. The fast evolution of vaccine antigen–encoding genes has serious consequences for the ability of current vaccines to continue to control pertussis.

Keywords. pertussis; genomics; evolution; vaccine.

Whooping cough, or pertussis, is caused primarily by the bacterium *Bordetella pertussis*. In England and Wales, 9711 laboratory-confirmed cases were recorded in 2012, leading to 14 deaths of infants <3 months of age. This was much greater than the previous recent peak year in 2008, in which 902 cases were reported, despite levels of vaccine coverage and diagnostic methods not changing during this period [1, 2]. Similar outbreaks have been reported across the globe [3], contributing to the consensus that pertussis is a resurgent disease that might be no longer effectively controlled by current vaccination programs.

Resurgence has been linked to increased surveillance, better diagnostic techniques, incomplete vaccination of populations, and, primarily, the switch from whole-cell pertussis vaccines (WCVs) to acellular pertussis vaccines (ACVs), which contains 1–5 purified *B. pertussis* protein antigens: pertussis toxin (Ptx), filamentous hemagglutinin (FHA), pertactin (Prn), and fimbrial type 2 (Fim2) and Fim3. In the United Kingdom (UK), a 5-antigen ACV has been used. ACV-induced immunity appears to be shorter lived than that induced by WCVs, possibly resulting in an expanded pool of carriers, particularly adolescents, and decreased herd immunity [4, 5]. In addition, studies using an infant baboon model revealed that, while ACVs protect the individual from disease symptoms, they are less able to prevent colonization of and transmission from the vaccinee, compared with WCVs. Increased transmission of *B. pertussis* in populations using ACVs, compared with those using WCVs, is proposed to contribute to the resurgence [6]. Finally, it has been proposed that vaccine-induced escape mutants are arising, because ACV-induced immunity is focused on just a few antigens and because changes in these antigens might result in strains that are less well recognized by this immunity [7].

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Correspondence: Andrew Preston, DPhil, Department of Biology and Biochemistry, University of Bath, Bath, BA2 7AY, UK (a.preston@bath.ac.uk).

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The frequency of different alleles of vaccine antigen–encoding genes among strains has changed over time [8–11]. The most common allelic profile among currently circulating strains (*ptxA1-ptxP3, prn2, fim3-2,* and *fim2-1*) is different from that of strains used for vaccine manufacture [12, 13], and isolates that do not express Prn are increasingly common [14–16]. *PtxP* refers to alleles of the *ptx* promoter. *PtxP3* is now dominant worldwide [17], and some studies suggest that *ptxP3* strains may have increased virulence, compared with *ptxP1* strains [18].

The study of genetic changes in *B. pertussis* over time was hindered by the high levels of homogeneity among *B. pertussis* and the lack of fine-resolution tools. Recently, the genome sequences of a large panel of *B. pertussis* strains collected from around the world and across many decades were generated and analyzed [19]. This provided detailed information about the population structure and evolution of *B. pertussis*, revealing significant genetic changes among strains over the last 50 years. A lack of geographical clustering of strains suggested rapid strain flow between countries. However, this panel of strains did not contain isolates collected more recently than 2008, except for 3 isolates from the Netherlands, which were collected in 2009 and 2010. In addition, the panel did not include a substantial number of isolates from a specific outbreak, leaving the genetic make-up of isolates involved in such events largely unknown. Here, we analyze a large panel of UK strains, with a focus on strains from the recent UK outbreak, to understand the clonal structure of the outbreak and determine whether there is evidence for vaccine-mediated immunity driving the evolution of these strains.

METHODS

Accession Numbers

Genome sequence data has been deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena/; Supplementary Table 1).

B. pertussis Strains

One hundred *B. pertussis* isolates were obtained from the National Reference Laboratory, Respiratory and Vaccine Preventable Bacteria Reference Unit, at Public Health England (Supplementary Table 1). Five strains were collected between 1920 and 1956 (hereafter referred to as the "prevaccine era"), 6 strains collected between 1957 and 2000 (hereafter, the "WCV era"), and 89 strains were collected between 2000 and 2012 (hereafter, the "ACV era"). Serotyping was performed using sera specific for antigens 1, 2, and 3 (89/596, 89/598, and 89/600, respectively; National Institute for Biological Standards and Controls, Potters Bar, UK) as previously described [12]. Tohama I (accession number: BX470248), a strain isolated in Japan in 1954, is the most widely studied strain, provides the reference genome sequence of *B. pertussis* [20], and is one of the strains used to produce ACVs used in the UK. *B. pertussis* isolates were grown on charcoal agar for 72 hours at 37°C.

DNA Preparation

Genomic DNA extraction was performed using the Qiagen DNA prep kit according to the manufacturer's instructions.

DNA Sequencing and Single-Nucleotide Polymorphism (SNP) Identification

Twenty four isolates were sequenced previously [19]. For the remainder, multiplex libraries, with fragment sizes between 300 bp and 500 bp, were prepared as previously described [21], with modifications [22]. Reads for each isolate were aligned to the Tohama I reference genome, using SMALT, version 0.7.4 (http://www.sanger.ac.uk/resources/software/smalt/). Base calls were made as previously described [21], using a combination of samtools, mpileup, and bcftools [23], allowing SNPs, and small insertions and deletions relative to Tohama I to be identified. Five strains produced poor-quality sequence and were excluded from the analysis, resulting in 95 strains being taken forward for analysis.

Phylogenetic Analysis

Maximum likelihood phylogenetic analysis was performed on variable sites from across the whole genomes, using RAxML under a general time-reversible evolutionary model and a Γ correction for among site rate heterogeneity [24]. One hundred random bootstrap replicates were run to provide support for relationships identified in the tree.

Analysis of SNP Densities

SNPs were reconstructed on to the phylogenetic tree using parsimony. SNP densities (defined as the number of SNPs per bp) within vaccine antigen–encoding genes (9 genes: *fhaB*, *prn*, *fim2*, *fim3*, and *ptxA–E*) or functional cell surface protein–encoding genes (591 genes, as categorized previously [20]) were calculated by counting the number of SNPs per bp of each gene. The difference between the mean per-gene SNP densities of vaccine antigen–encoding genes and cell surface protein–encoding genes was calculated. The significance of this difference was calculated using a nonparametric Monte Carlo simulation. In our randomizations of all the data, preserving relative sample sizes, it was recorded how often a difference as large, or greater than the difference above, was observed, by repeated randomly resampling 2 samples of the same size as above. Under this protocol, if n is the number of observations that have greater than or equal to the observed difference in SNP density and m is the number of simulations (in this case, 10 000), then $P = [n + 1]/[m + 1]$ is the unbiased estimator of the type I error rate.

We performed a similar procedure to compare SNP densities in vaccine antigen–encoding genes between eras. To account for

differences in SNP densities between strains from the different eras, the SNP densities of the vaccine antigen–encoding genes were normalized with respect to the SNP densities of all the genes considered (vaccine antigen–encoding and surface protein–encoding genes). A nonparametric Monte Carlo simulation compared the normalized SNP densities in the ACV antigen genes in ACV-era strains with those for pre–ACV-era strains, with *P* determined as described above.

Allele Typing

The different alleles of *prn*, *ptxA*, *ptxP*, *fim3*, and *fim2* genes have been previously described [11] and were used to identify allele types from DNA sequences.

Analysis of *prn* From UK50

The *prn* locus was amplified from UK50 by polymerase chain reaction (PCR) analysis, using primers 5′-CCGCTGATTCGC CACAAG-3′ and 5′-GTGCGGTACTTGCCCTTG-3′. PCR products were cloned using the Gateway system (Invitrogen, Paisley, UK) and sequenced by Eurofins Genomics (Ebersberg, Germany), using standard M13 forward and reverse primers and internal primers 5′-GCGCACGCCTGTCCAAAG-3′ and 5′-TAGCGAGCCAGCACGTAG-3′.

Analysis of Differences in DNA Content Among Strains

To detect gene loss from strains, compared to the DNA content of Tohama I, coverage plots generated using the paired-end reads mapped to this reference genome were used to create a heat map. DNA sequence contigs that did not map to Tohama I were analyzed using Blastn and Blastx (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Phylogeny of UK Strains From 1920 to 2012

Phylogenetic analysis based on SNPs across the whole-genome sequences was performed to understand the evolutionary relationships between the UK strains analyzed (Figure 1 and Supplementary Table 1). Strains isolated during 1920–1982 form a cluster and are generally separated from strains isolated during 2008–2012. The most distinct clustering separates strains carrying the *ptxP1* allele from those carrying the *ptxP3* allele, which, as found elsewhere, is the predominant *ptxP* type among recent strains. This phylogenetic analysis was extended to place the UK strains in the global phylogenetic tree described elsewhere (Figure 2) [19]. This reveals that the UK *ptxP3* strains separate into 2 clusters distinguished by the presence of the *fim3-2* allele. The UK outbreak strains largely cluster with strains isolated mainly during the early 2000s from a variety of geographical areas, including North America, Europe, and Australia.

Vaccine Antigen Allele Profiles

Previously, *ptxP3-ptxA1-prn2-fim3-2* was defined as the dominant allele type circulating in the UK and other countries [17]. Typing of alleles among the outbreak strains reveal no recent change in this profile (Table 1). Numerous isolates deficient for the production of Prn have been reported in other countries, and a number of different mutations in *prn* responsible for this phenotype identified [14–16]. It has been suggested that loss of Prn expression has been selected by vaccine-mediated immunity pressure. Interestingly, just a single UK strain, UK50, was mutated for *prn*. This was identified by a lack of sequence reads mapping to a region of *prn*. The *prn* locus was amplified

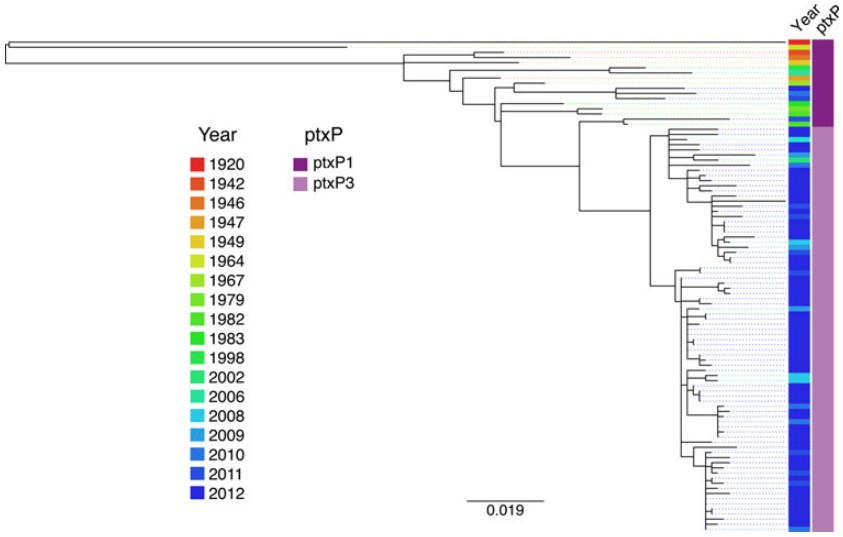

Figure 1. Phylogenetic tree depicting the evolutionary relationships among the United Kingdom (UK) *Bordetella pertussis* isolates studied here. Maximum likelihood phylogenetic analysis was performed on variable sites from across the whole genomes, using RAxML. Strains are shaded according to their year of isolation and *ptxP* type.

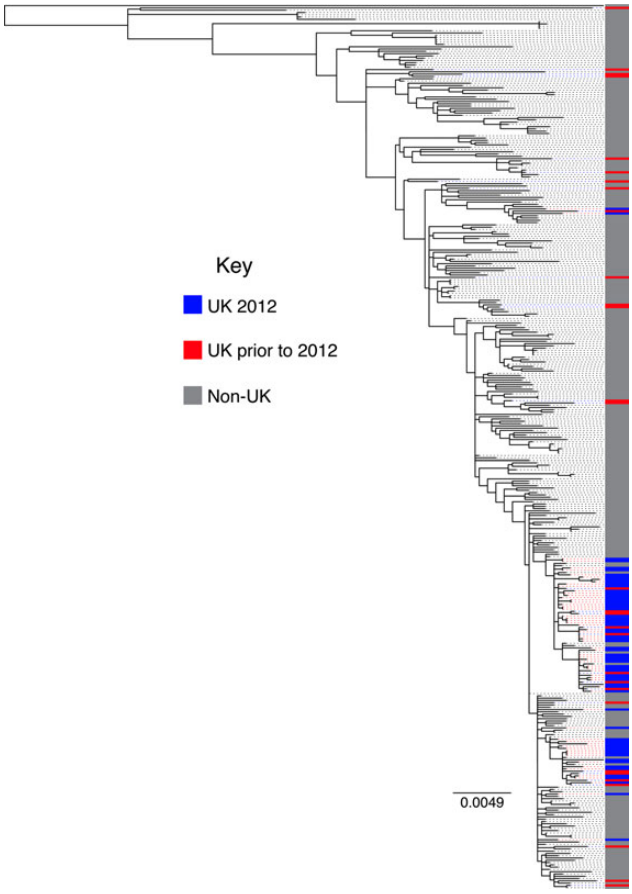

Figure 2. Phylogenetic relationships of United Kingdom (UK) strains within a global context. The UK isolates analyzed here are indicated.

Table 1. Frequency of Vaccine Antigen–Encoding Gene Alleles Among United Kingdom Strains, by Vaccine Era

Variable	Prevaccine Era, 1920–1956 (n = 5)	WCV Era, 1957–2000 (n = 6)	ACV Era, 2001–2012 (n = 84)
ptxP			
1	100	100	6
3	0	0	94
ptxA			
1	20	100	100
2	80	0	0
Prn[a]			
1	100	84	5
2	0	0	91
3	0	16	3
4	0	0	1
Fim2-			
1	100	100	100
Fim3-			
1	100	100	70
2	0	0	29
3	0	0	1
Serotype			
1[b]	20	0	0
1, 2	40	50	37
1, 3	20	17	63
1, 2, 3	20	33	0

Data are percentage of strains tested.

Abbreviations: ACV, acellular pertussis vaccine; WCV, whole-cell pertussis vaccine.

[a] Prn allele type was determined for just 76 ACV era strains, owing to poor mapping of reads in this region in 8 strains.

[b] Serotype was not determined for one ACV era strain. Thus, frequencies are based on 83, not 84, strains in this era.

by PCR from this strain, and the resulting product was sequenced using Sanger sequencing. This identified that a recombination event between 2 copies of IS1663 resulted in a deletion/insertion mutation in which the 5′ 1326-bp region of the *prn* coding sequence was deleted. Aberrant mapping was not observed for any other UK strain. In other countries, a common *prn* mutation arose from insertion of IS481 into *prn*. We identified paired-end reads in which one read mapped within IS481 but the other did not and, thus, derives from the region flanking IS481. Mapping these reads to the reference genome identified the position of the copies of IS481 within each query strain. No IS481 insertions into *prn* were identified among UK strains. It is not clear why so few Prn-deficient strains, compared with other countries experiencing pertussis outbreaks, have been identified in the UK.

SNPs Specific to *ptxP3* Strains

ptxP3 strains are the predominate type in current circulation and appear to have infection-associated biologic characteristics that differ from those of *ptxP1* strains. The *ptxP3* SNP appears to be both a direct cause and a marker for other genetic variations that contribute to this difference [18]. To investigate the genetic traits of UK *ptxP3* strains, SNPs specific to this lineage were identified. In total, 22 such SNPs were identified (Table 2). Ten were intergenic, of which 7 were in the direct repeat region of IS elements, which are present in multiple copies in the *B. pertussis* genome. It is not clear whether these particular IS elements are functional. Twelve SNPs were in coding regions. Of those, 7 were nonsynonymous mutations (NSMs) and 5 were synonymous mutations (SMs). The 7 NSMs were in genes within the functional categories "transport/binding proteins," "conserved hypothetical," "pathogenicity," "unknown," and "regulation" or as "pseudogenes" as previously described [20]. All of these SNPs were also identified among the global panel of *B. pertussis* strains [19]. However, of the 22 SNPs identified here as being *ptxP3* specific, only 10 were identified as being *ptxP3*-type specific in the previous study (Table 2); the other 12 SNPs were also identified among non-*ptxP3* strains globally.

Table 2. Single-Nucleotide Polymorphisms (SNPs) Specific to United Kingdom *ptxP3* Strains

Location[a]	Type	Mutation	Global *ptxP3*[b]	Details
36 857	INT	A→G	Yes	93 bp upstream of BP0032 (encoding a putative transport protein), 156 bp upstream of BP0033 (encoding GlyQ-glycyl-tRNA synthetase α chain)
617 083	INT	T→G	No	Within the 5′ repeat region of IS481 (BP0611), 31 bp upstream of transposase start codon.
617 084	INT	C→T	No	Within the 5′ repeat region of IS481 (BP0611), 32 bp upstream of transposase start codon.
1 077 844	INT	C→T	No	Within the 5′ repeat region of IS1663 (BP1035), 139 bp upstream of transposase start codon.
1 170 424	INT	A→G	No	Within the 5′ repeat region of IS481 (BP1114), 31 bp upstream of transposase start codon.
1 222 400	INT	A→C	No	Within the 5′ repeat region of IS481 (BP1157), 31 bp upstream of transposase start codon.
1 635 654	INT	T→G	No	Within the 5′ repeat region of IS481 (BP1557), 31 bp upstream of transposase start codon.
2 259 917	INT	G→C	No	Within the 5′ repeat region of IS481 (BP2135), 98 bp upstream of transposase start codon.
3 263 622	INT	A→C	Yes	193 bp away from BP3062, putative integral membrane transport protein.
3 988 168	INT	G→A	Yes	89 nucleotides away from the start codon of ptxA, ptxP3allele
196 307	NSM	T→C	Yes	BP0194, putative transport protein
299 559	NSM	C→T	Yes	BP0292, pseudogene, conserved hypothetical protein
1 331 840	NSM	G→A	Yes	Pseudogene, BP1261, hypothetical protein
1 547 488	NSM	A→G	No	BP1471, conserved hypothetical protein
2 374 322	NSM	T→C	Yes	BP2249, BscI, type III secretion apparatus protein
2 651 008	NSM	G→A	Yes	BP2502, Hypothetical protein
3 134 458	NSM	G→C	No	BP2946, probable transcriptional regulator
185 405	SM	G→A	No	BP0184, putative periplasmic protein
518 837	SM	T→C	No	BP0507, putative membrane protein
694 521	SM	A→G	Yes	BP0678, Putative peptide chain release factor
3 840 411	SM	G→A	Yes	BP3630, RpsH, 30S ribosomal protein
3 991 376	SM	C→T	No	BP3787, PtxC, Pertussis toxin subunit protein

Abbreviations: INT, SNP is in an intergenic region; NSM, nonsynonymous mutation; SM, synonymous mutation.

[a] Tohama I reference genome coordinates (accession no. BX470248).

[b] "Yes" denotes that the SNP was also defined as *ptxP3* specific in study of the global *Bordetella pertussis* population [1], whereas "No" denotes that the SNP was not defined as such in the study.

SNP Rates Are High in Vaccine Antigen–Encoding Genes

Previously, it was identified that genes encoding functional cell surface proteins had higher SNP densities than the *B. pertussis* chromosomal average [19]. However, ACV-mediated immunity is exerting selective pressure primarily on the proteins used in these vaccines and might be driving their evolution. To explore this, the SNP density for the 9 ACV antigen-encoding genes (Ptx comprises 5 different proteins) and for the other 591 genes composing the cell surface protein category was calculated for all strains within each vaccine era and compared. Second, we investigated whether the SNP rate in ACV antigen–encoding genes had increased since the introduction of ACVs.

The difference in mean SNP density across genes within the 2 samples (calculated as the mean SNP density in vaccine antigen–encoding genes minus the mean SNP density in cell surface protein–encoding genes) was calculated. A nonparametric Monte Carlo simulation was used to assess the significance of this difference by determining how often a difference as large or larger than this was derived by randomly resampling, from the pool of vaccine antigen–encoding and cell surface protein–encoding genes, 2 samples of the same size as the 2 samples described above. This revealed that, in each era, vaccine antigen–encoding genes had significantly higher SNP densities than other cell surface protein–encoding genes ($P < .05$; Table 3), with the difference being greatest among ACV-era strains. This suggests that the vaccine antigen–encoding genes are faster evolving than other surface protein–encoding genes and that they were also faster evolving even before the introduction of widespread vaccination.

To compare SNP densities in vaccine antigen–encoding genes between eras, SNP densities within each era were normalized by dividing by the mean SNP rate across all of the genes concerned (ie, those encoding ACV antigens and cell surface proteins). In comparison to the prior analysis, this has less power, owing to the much smaller sample of ACV antigen–encoding genes, compared with the total number of cell surface protein–encoding genes. Although the normalized SNP density in ACV-era strains was greater than in pre–ACV era strains, the difference was not statistically significant ($P = .160$). However, the number of pre-ACV strains in this analysis was small. Thus, the same analyses were repeated, using SNP data from the global collection of strains for which the year of isolation

Table 3. Single-Nucleotide Polymorphism (SNP) Rates in Vaccine Antigen–Encoding Genes, Compared With Other Cell Surface Protein–Encoding Genes, Among Strains Isolated in the United Kingdom and Globally During Different Vaccine Eras

Strain Source, Era	Strains, No.	SNPs/bp, No., Mean		Difference[a]	Normalized Difference[b]	*P* Values[c]
		Vaccine Antigen–Encoding Genes	Cell Surface Protein-Encoding Genes			
United Kingdom						
Prevaccine era, 1920–1956	5	3×10^{-4}	7.8×10^{-5}	2.22×10^{-4}	2.72	.045
WCV era, 1957–2000	6	4.75×10^{-4}	5.9×10^{-5}	4.17×10^{-4}	6.40	.016
ACV era, 2001–2012	84	1.73×10^{-3}	1.55×10^{-4}	1.57×10^{-3}	8.82	.0004
Global						
Prevaccine era, 1920–1956	19	1.45×10^{-3}	5.85×10^{-4}	8.62×10^{-4}	1.44	.012
WCV era, 1957–2000	204	2.62×10^{-3}	1.01×10^{-3}	1.61×10^{-3}	1.56	.002
ACV era, 2001–2012	188	2.91×10^{-3}	4.23×10^{-4}	2.49×10^{-3}	5.41	.0001

Abbreviations: ACV, acellular pertussis vaccine; WCV, whole-cell pertussis vaccine.

[a] Calculated as the mean SNP density in vaccine antigen–encoding genes minus the mean SNP density in cell surface protein–encoding genes.

[b] Calculated as [the mean SNP density in vaccine antigen–encoding genes minus the mean SNP density in cell surface protein–encoding genes]/mean overall SNP density.

[c] For the difference in SNP rate between vaccine antigen–encoding genes and cell surface protein–encoding genes.

was known [19] and incorporating the UK strains sequenced here (Table 3). Again, a significantly greater SNP frequency was found in ACV antigen–encoding genes, compared with other cell surface protein–encoding genes, in all 3 eras. This time, there was also a significantly higher SNP frequency in ACV antigen–encoding genes among ACV era strains, compared with pre–ACV era strains ($P = .0177$), suggesting that the relative SNP density in ACV antigen–encoding genes has increased since the introduction of ACVs. These results suggest that ACV antigen–encoding genes are intrinsically fast evolving and provide some support for the hypothesis that they have evolved even faster since the introduction of ACVs.

The more rapid evolution in the ACV antigen–encoding genes could be due to either a higher underlying mutation rate or different selection at the protein level. The different selection could be positive selection or weaker purifying selection. To distinguish between these 2 possibilities, SNPs were split into SMs and NSMs. High NSM but not SM rates would suggest altered protein-level selection. A higher rate of synonymous evolution (with possibly a weak nonsynonymous effect) would suggest higher mutation rates. Interpretation here is difficult, owing to well-described but incompletely understood correlation between SM and NSM rates.

Among WCV- and ACV-era global strains but not prevaccine era strains, the SM frequency was significantly higher in ACV antigen–encoding genes, compared with other cell surface protein–encoding genes (Table 4). When comparing ACV-era strains to pre-ACV era strains, the SM frequency in ACV antigen genes was significantly higher ($P = .004$). NSMs also occurred at a significantly greater frequency in ACV antigen–encoding genes, compared with other cell surface protein–encoding genes (Table 4). The magnitude of this effect is greater than that seen for SMs, suggesting the higher evolutionary rate of ACV antigen–encoding genes, compared with cell surface protein–encoding genes, is largely owing to protein-level selection on the antigens. Evidence for a strong recent increase is less clear-cut. When comparing strains from the ACV-era to pre-ACV era strains, the NSM frequency in ACV antigen genes was on the edge of significance ($P = .051$). Overall, our results provide support for the hypothesis that the genes encoding antigens chosen for ACVs are intrinsically fast evolving, in part owing to selection pressure on their antigenic products. We cannot discount the possibility that, in the ACV era, there has been an increase in the mutation rate (see "Discussion" section).

Regions of Difference

Deletions have been a major feature of *B. pertussis* evolution and appear to be ongoing [20, 25]. Compared with the Tohama I reference genome, most of the major deletions observed among the strains analyzed here had been identified previously [25]. Numerous small deletions were found in only a few isolates or just 1 isolate, suggesting that deletion of DNA is common among *B. pertussis* strains. Interestingly, some deletions appeared to be specific to the UK *ptxP3* strains, but no deletions specific to outbreak isolates were detected (Supplementary Figure 1).

Regions from individual strains that were not present in the Tohama I reference genome were investigated by BLAST analyses. These regions were also found within other *B. pertussis* genomes (BP18323 and CS) or in *Bordetella bronchiseptica* RB50, similar to that reported in other studies [26]. Thus, there were no novel insertions or gene acquisition among outbreak isolates.

Table 4. Synonymous and Nonsynonymous Mutation Rates in Vaccine Antigen–Encoding Genes, Compared With Other Cell Surface Protein–Encoding Genes, Among Strains Isolated Globally During Different Vaccine Eras

Mutation Type, Era	Strains, No.	SNPs/bp, No., Mean		Difference[a]	Normalized Difference[b]	*P* Values[c]
		Vaccine Antigen–Encoding Genes	Cell Surface Protein–Encoding Genes			
Synonymous						
Prevaccine era, 1920–1956	19	1.32×10^{-4}	2.4×10^{-4}	-1.07×10^{-4}	−0.45	.627
WCV era, 1957–2000	204	9.66×10^{-4}	4.23×10^{-4}	5.43×10^{-4}	1.26	.045
ACV era, 2001–2012	188	9.68×10^{-4}	1.76×10^{-4}	7.92×10^{-4}	4.20	.011
Nonsynonymous						
Prevaccine era, 1920–1956	19	1.18×10^{-3}	3.40×10^{-4}	8.38×10^{-4}	2.38	.006
WCV era, 1957–2000	204	1.96×10^{-3}	5.83×10^{-4}	1.37×10^{-3}	2.28	.002
ACV era, 2001–2012	188	1.95×10^{-3}	2.38×10^{-4}	1.71×10^{-3}	6.48	.0002

Abbreviations: ACV, acellular pertussis vaccine; SNP, single-nucleotide polymorphism; WCV, whole-cell pertussis vaccine.

[a] Calculated as the mean SNP density in vaccine antigen–encoding genes minus the mean SNP density in cell surface protein–encoding genes.

[b] Calculated as [the mean SNP density in vaccine antigen–encoding genes minus the mean SNP density in cell surface protein–encoding genes]/mean overall SNP density.

[c] For the difference in SNP rate between vaccine antigen–encoding genes and cell surface protein–encoding genes.

DISCUSSION

The resurgence of pertussis in countries with high levels of vaccination has caused widespread concern. Among other factors, *B. pertussis* evolution away from efficient control by vaccine-induced immunity has been proposed as a contributor to this. Recently, whole-genome sequencing was used to define global genetic variability among *B. pertussis* isolates, and it identified genetic changes in the *B. pertussis* population over time [19].

Here, we have analyzed in detail the genomes of UK *B. pertussis* isolates, with an emphasis on strains from the 2012 outbreak. We are the first to show that many genetically distinct *B. pertussis* strains contributed to this outbreak and, importantly, that it was not due to the emergence of a novel, hypervirulent clone or to expansion of an individual lineage. Furthermore, outbreak strains were genetically very similar to those circulating during periods when the incidence of pertussis was low.

The *ptxP3* type is the dominant clone worldwide, and UK outbreak strains are also predominantly of this type. Analysis of global isolates identified just 19 SNPs as being *ptxP3* specific [19]. Here, 22 SNPs distinguished *ptxP3* strains from *ptxP1* strains. However, just 10 of these were common to both sets of *ptxP3*-specific SNPs. If *ptxP3* strains have increased fitness or virulence than older isolates, our analysis suggests that very few SNPs are responsible for this, or that particular combinations of SNPs are important, only some of which are *ptxP3* specific. Overall, these data argue against large-scale genetic changes being behind the recent resurgence in pertussis.

Changes in alleles of the genes encoding vaccine antigens have been well documented [27], and these findings support the hypothesis that selection pressure from ACV-induced immunity is a driver of *B. pertussis* evolution. However, definitive studies to demonstrate that allelic variation enhances evasion of vaccine-mediated immunity are lacking and particularly difficult to perform, because of the inability to conduct studies with human hosts, and studies using animal models struggle to detect subtle changes and will not include population-level effects that are certainly important for selection of variants among *B. pertussis* worldwide. Here, we provide compelling evidence that genes encoding ACV antigens are evolving more rapidly than other cell surface protein–encoding genes (which we consider the most suitable comparator group), containing a significantly higher frequency of SNPs in each of the vaccine eras. Interestingly, this was true even in the prevaccine era. It is likely that, even in the absence of vaccination, the natural immune response to these antigens creates selective pressure, particularly for a pathogen that is restricted to the human respiratory tract. Of particular importance is that we calculated that ACV antigen–encoding gene evolution rates have increased significantly since the introduction of ACVs, the first demonstration of this effect. This might suggest that the use of ACVs has increased selection pressure on ACV antigens, selecting for ACV antigen–encoding gene variants. However, we also calculated that, while the frequency of SMs in ACV antigen–encoding genes was significantly higher in ACV-era strains, compared with older strains, the frequency of NSMs was on the edge of significance ($P = .051$). In turn, this suggests that selection pressure from vaccine-mediated immunity is not the sole driving force for ACV antigen–encoding gene variation. A different interpretation is that the mutation rate of ACV antigen–encoding genes has increased since the introduction of ACVs. If synonymous sites are under weak purifying selection pressure (ie, not perfectly neutral), then there is a lag between a SNP arising and its elimination by this selection, resulting in an

excess of SNPs in the modern era. However, normalizing the ACV antigen–encoding gene SNP rates by the SNP rates for all genes within the era largely eliminates this effect (ie, SMs in cell surface protein–encoding genes should be equally overrepresented in the modern era). However, if SMs in ACV antigen–encoding genes and cell surface protein–encoding genes are under different intensities of purifying selection, then our result could be found.

Either way, the more rapid evolution at the protein level (as determined by NSMs) of ACV proteins, compared with other cell surface proteins, across all eras suggests that strains will become increasingly mismatched to those used for vaccine production, and this could lead to decreased vaccine efficacy over time. The ACV antigens were chosen on the basis of their immunogenicity, but it could be that this property has driven the relatively high evolution rates of the genes encoding these antigens. Our results raise fresh concerns over the ability of current ACVs to continue to control disease.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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